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(54) Title: PRODUCTION OF C-TERMINAL AMIDATED PEPTIDES FROM RECOMBINANT PROTEIN CONSTRUCTS

(57) Abstract

A method for the production of C-terminal amidated recombinant peptides is provided. The method employs a recombinant protein construct having multiple copies of a target peptide linked by intraconnecting peptides. The intraconnecting peptides permit the multicopy construct to be selectively reacted to produce product peptides having a C-terminal α-carboxamide. A recombinant gene containing a DNA sequence coding for the recombinant protein construct and an expression cassette, an expression vector and a transformed cell including the recombinant gene are also provided.

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PRODUCTION OF C-TERMINAL AMIDATED PEPTIDES FROM RECOMBINANT PROTEIN CONSTRUCTS

Background of the Invention

In vitro DNA manipulation and the attendant transfer of genetic information have developed into a technology that allows the efficient expression of endogenous and foreign proteins in microbial hosts. Recombinant DNA techniques have made possible the selection, amplification and manipulation of expression of proteins and peptides. For example, changes in the sequence of the recombinantly produced proteins or peptides can be accomplished by altering the DNA sequence by techniques like site-directed or deletion mutagenesis.

Some modifications to a recombinantly produced protein or peptide, however, cannot be accomplished by altering the DNA sequence. For example, while the C-terminal α-carboxyl group in many naturally occurring protein and peptides often exists as an amide, this amide typically is not produced directly through expression. Rather, a precursor protein is produced by expression and the amide is biologically produced *in vivo* from the precursor protein.

Moreover, although expression of any foreign protein in any microbial host is theoretically possible, the stability of the protein produced often limits such practice and results in a low yield. In particular, small foreign proteins and oligopeptides cannot be overproduced in most cellular hosts. Expression of a small peptide in a host cell raises the possibility that the host will assimilate the peptide. For example, where size of the desired peptide is no more than about 60 to 80 amino acid units in length, degradation usually occurs rather than end product accumulation.

In response to this problem, small peptides have been expressed as part of fusion proteins which include a second larger peptide, such as a marker peptide (e.g., β-galactosidase or chloramphenicol acetyl transferase). While the use of a fusion protein may avoid

assimilation, this approach may lead to other problems. Purification is often not very efficient or effective. Many of the marker peptides are such large molecular weight proteins that the desired protein constitutes only a small fraction of the fusion protein.

Another approach involves the expression of a recombinant construct which includes multiple copies of the desired peptide (a multicopy construct). The multicopy construct may or may not include a marker peptide or other leader sequence. Typically, the multicopy construct is designed so that the molecular weight is sufficient to prevent assimilation by the host cell.

The multicopy approach has typically been carried out with methionine residues positioned between the desired peptides. While such constructs can be selectively cleaved with cyanogen bromide, the use of multicopy construct with methionine cleavage sites is limited to the production of product peptides which lack a methionine residue (Met). In addition, cleavage of a multicopy construct at a methionine produces peptides with a C-terminal homoserine (Hse) lactone. This unnatural amino acid residue can be converted to the free acid or amide by ring opening. The amidated peptide, however, contains the unnatural amino acid residue, homoserine, as its C-terminal residue. Thus, known multicopy based methods which make use of methionine as a cleavage site do not permit the production of α-amidated forms of native peptide sequences.

Other reports of multicopy-based peptide production disclose the use of an acid sensitive cleavage site, -Asp-Pro-, or a tripeptide linker sequence which is cleaved by a specific pair of proteases (trypsin and α-chymotrypsin). Neither of these methods, however, permits the generation of the α-amidated form of

peptides without placing some limitation on the amino acid sequence of the product peptide.

Accordingly, there is a continuing need for efficient flexible, inexpensive and convenient methods for the recombinant production of C-terminal amidated peptides. In particular, there is a need for methods which permit the production of a recombinant peptide in its α-amidated form without any limitations as to the amino acid sequence of the peptide. It is therefore an object of the present invention to provide an improved method for the production of a recombinantly produced C-terminal α-amidated peptide. A further object is to provide a simple and efficient method for modification of a recombinantly produced peptide which permits the exchange of an unnatural C-terminal homoserine residue with the amidated form of another amino acid. These and other objects are accomplished by the present invention.

Summary of the Invention

The present invention provides a method for the production of C-terminal amidated recombinant peptides regardless of their sequence. The method allows the efficient production of peptides which cannot normally be obtained through recombinant technology. Typically, a recombinant protein construct having multiple copies of a target peptide is employed. The target peptide units are linked by intraconnecting peptides which permit the multicopy construct to be selectively reacted to produce product peptides having a C-terminal α-carboxamide. The recombinant protein construct may also include a adjunct peptide. The adjunct peptide generally is located near the N-terminus of the construct.

In one embodiment of the invention, the multicopy construct is cleaved to directly produce product peptides having a C-terminal α-carboxamide. In another embodiment, the multicopy construct is cleaved to

precursor peptides which can be modified in a controlled manner to generate the desired C-terminal α-amidated product peptides.

Target peptides free of methionine residues may be produced using the present method. Target peptides of this type may be produced from a multicopy construct having intraconnecting peptides which include a methionine residue. Where the methionine residue is directly linked to the C-terminus of the target peptide, the multicopy construct may be cleaved with cyanogen bromide. The resulting fragments may be transpeptidated using a carboxypeptidase, e.g., a serine carboxypeptidase such as carboxypeptidase Y, to replace the C-terminal homoserine residue with an α-amidated amino acid. The fragments may be also transamidated with the carboxypeptidase to replace the C-terminal homoserine residue with a 2-nitrobenzylamine compound. This produces a fragment having a C-terminal (2-nitrobenzyl)amido group which may be photochemically decomposed to produce an α-amidated peptide fragment minus the homoserine residue.

The present method is particularly suitable for producing peptides from a recombinant protein construct including at least two copies of a target peptide free of unblocked cysteine residues. The target peptides are preferably linked by intraconnecting peptides which include a cysteine residue. If the cysteine residue is directly adjacent the C-terminus of the target peptide, the construct may be cleaved by an aminolysis reaction to a first α-amidated peptide. This is achieved by reacting the cysteine residue with an S-cyanylating agent to form an S-derivatized cysteine residue (activation) and reacting the S-derivatized cysteine residue with an amino compound (aminolysis). More preferably, the intraconnecting peptides include a second cleavage site which permits the N-terminal

residues of the first α-amidated peptide to be cleaved to produce a desired α-amidated product peptide.

Another embodiment of the invention provides a recombinant protein construct which includes an amino acid sequence of the formula:

Yyy-(CS1)-TargP-(Cys)-Xxx

wherein the Yyy- is a leader group, -(CS1)- is a cleavage site, the -TargP- is a target peptide and -Xxx is a tail group. The target peptide and the -(CS1)- cleavage site are free of unblocked cysteine residues.

C-terminal α-amidated peptides may also be produced by the present method from a multicopy construct containing copies of a target peptide which includes both a methionine residue and a cysteine residue. Furthermore, the C-terminal α-amidated peptide may be produced by simultaneously cleaving and transpeptidating with an endopeptidase.

Brief Description of the Figures

FIG. 1 depicts a portion of plasmid pBN1:PTH(1-34)C-1_c (SEQ ID NO:1) (and corresponding amino acid sequence (SEQ ID NO:2)) coding for a fusion protein construct containing a single copy of PTH(1-34) (SEQ ID NO:56).

FIG. 2 depicts a portion of plasmid pBN1:PTH(1-34)C-2_c (SEQ ID NO:3) (and corresponding amino acid sequence (SEQ ID NO:4)) coding for a fusion protein construct containing two copies of PTH(1-34) (SEQ ID NO:56).

FIG. 3 depicts a portion of plasmid pBN2:GRF(1-44)C-1_c (SEQ ID NO:5) (and corresponding amino acid sequence (SEQ ID NO:6)) coding for a fusion protein construct containing a single copy of GRF(1-44) (SEQ ID NO:57).

FIG. 4A-B depicts a portion of plasmid pBN2:GRF(1-44)C-2_c (SEQ ID NO:7) (and corresponding amino acid sequence (SEQ ID NO:8)) coding for a fusion protein

construct including two copies of GRF(1-44) (SEQ ID NO:57).

FIG. 5 depicts a portion of plasmid pBN1:GLP(7-36)C-1_c (SEQ ID NO:9) (and corresponding amino acid sequence (SEQ ID NO:10)) coding for a fusion protein construct including a single copy of GLP1(7-36) (SEQ ID NO:53).

FIG. 6A-B depicts a portion of plasmid pBN1:GLP(7-36)C-2_c (SEQ ID NO:11) (and corresponding amino acid sequence (SEQ ID NO:12)) coding for a fusion protein construct including two copies of GLP1(7-36) (SEQ ID NO:53).

FIG. 7 depicts the various formulas for a portion of the fusion protein construct formed of multiple units of target peptides.

Detailed Description of the Invention

The present method of producing a C-terminal α-amidated peptide typically includes cleaving a recombinant protein construct which includes an amino acid sequence of the formula:

$$\text{Yyy-TargP'-(CS2)-[-(Ln1)}_n\text{-(CS1)}_m\text{-TargP-(CS2)-]}_r\text{-Xxx}$$

where -CS1- and -CS2- are cleavage sites, -(Ln1)- is a linking peptide, -TargP'- and -TargP- are a target peptide, n and m are 0 or 1, and r is an integer from 1 to about 150. The Yyy- is a leader group and -Xxx is a tail group. The -CS2- cleavage site may be either an enzymatic or chemical cleavage site. In a preferred embodiment of the invention, the -CS2- cleavage site is either a methionine residue or an unblocked cysteine residue and the target peptide is free of at least one amino acid residue selected from the group consisting of a methionine residue and an unblocked cysteine residue.

The size of the recombinant protein construct will vary depending on the nature and number of copies of the

target peptide. The recombinant protein construct is large enough to avoid degradation by the host cell (e.g., at least about 60 to 80 amino acid residues) and not so large that it can not be effectively expressed by the host cell. As a practical matter, the recombinant protein construct will have a molecular weight of up to about 500,000 although larger constructs are also within the scope of the present invention. The size of the recombinant protein construct is chosen such that it may be expressed by the host cell so as to avoid introducing errors in the protein sequence. This places practical limitations on the number of copies of the target peptide present in a given construct. The actual number will vary depending on the size and nuture of a particular target peptide within the limitations set by the factors discussed above.

The linking peptide may have one of a number of forms. In the simplest form, the linking peptide functions as a spacer unit. In another form, the linking peptide may include a additional peptide segment (a second target peptide). A third form is a single unit composed of several (i.e., two or more) identical or different peptide segments tandemly interlinked together by innerconnecting peptides. Yet another form is composed of repeating multiple tandem units linked together by connecting peptides wherein each unit contains the same series of different individual target peptides joined together by innerconnecting peptides. The innerconnecting peptides may include a cleavage site which may be the same or different from cleavage sites present in the interconnecting peptides or intraconnecting peptides. The forms described above are merely examples which illustrate the variations and modifications which may be made in the linking peptide (see Figure 7 for schematic depiction of protein constructs formed of multiple units of target peptides).

The target peptide may incorporate all or a portion of any natural or synthetic peptide desired as a product, e.g., any desired protein, oligopeptide or small molecular weight peptide. For the purposes of this application a peptide includes at least two amino acid residues linked by a peptide bond. Suitable embodiments of the target peptide include caltrin, calcitonin, insulin, tissue plasminogen activator, growth hormone, growth factors, growth hormone releasing factors, erythropoietin, interferons, interleukins, oxytocin, vasopressin, ACTH, collagen binding protein, glucagon like peptides, glucagon, parathyroid hormone, angiotensin, individual heavy and light antibody chains, individual chain fragments especially such as the isolated variable regions (VH or VL) as characterized by Lerner, <u>Science</u>, <u>246</u>, 1275 et. seq. (Dec. 1989) and epitopal regions such as those characterized by E. Ward et al., <u>Nature</u>, <u>341</u>, 544-546 (1986) wherein the antibodies, chains, fragments and regions have natural or immunogenetically developed antigenicity toward antigenic substances. Additional embodiments of the desired peptide include peptides having physiologic properties, such as sweetening peptides, mood altering peptides, nerve growth factors, regulatory proteins, functional hormones, enzymes, DNA polymerases, DNA modification enzymes, structural peptides, peptide analogs, neuropeptides, peptides exhibiting effects upon the cardiovascular, respiratory, excretory, lymphatic, immune, blood, reproductive, cell stimulatory and physiologic functional systems, leukemia inhibitor factors, antibiotic and bacteriostatic peptides (such as cecropins, attacins, apidaecins), insecticidal, herbicidal and fungicidal peptides as well as lysozymes.

The leader group (Yyy) includes at least one amino acid residue. The leader group may also include a peptide, e.g., an adjunct peptide, or a cleavage site. In a preferred embodiment of the invention, the leader

group includes a ligand binding protein, a highly charged peptide, an antigenic peptide, a polyhistidine-containing peptide, a hydrophobic peptide, or a DNA binding peptide. In another preferred embodiment of the invention, the leader group includes a cleavage site connected to the N-terminus of the -TargP'- target peptide.

The tail group (Xxx) may be a hydrogen or may include an amino acid residue or a peptide such as the adjunct peptide. Typically, the tail group includes a single amino acid or a short sequence of amino acids (e.g., up to about 10 amino acid residues). This facilitates the insertion of restriction enzyme sites into a recombinant DNA construct coding for the recombinant protein construct.

The recombinant protein construct may also include a adjunct peptide. The adjunct peptide may be included as part or all of either the leader group (Yyy) or the tail group (Xxx). Typically, the adjunct peptide is located near the N-terminus of the construct. The adjunct peptide may aid in preventing the assimilation of the construct by the host cell during expression and may also facilitate the isolation and/or purification of the construct. The adjunct peptide may include a ligand binding protein, a highly charged peptide, an antigenic peptide, a polyhistidine-containing peptide, a hydrophobic peptide, or a DNA binding peptide. All of these types of adjunct peptides allow the recombinant protein construct to be selectively removed from other cellular components. In a preferred version of the invention, the adjunct peptide includes a carbonic anhydrase (e.g., human carbonic anhydrase) or a modified functional version thereof. Suitable carbonic anhydrase adjunct peptides and their modified functional versions are described in International Application PCT/US91/04511, the disclosure of which is herein incorporated by reference.

The fusion protein construct may include a chemical cleavage site or an enzymatic cleavage site. The cleavage site or sites which may be incorporated into the fusion protein construct will depend upon the identity of the target peptide(s) present. The cleavage site and target peptide are typically selected so that target peptide does not contain an amino acid sequence corresponding to the cleavage site. Secondary considerations will also influence the choice of a particular cleavage site. In some instances, the cleavage sites may be designed so as to avoid the use of a enzymatic cleavage reaction. This may be accomplished by employing a chemical cleavage site, such as a site which may by cleaved after treament with an S-cyanylating agent (e.g, 2-nitro-5-thiocyanatobenzoate) or by treatment with an acid having a pK_a of no more than about 3.0. In other instances, it may be desireable to employ a cleavage site which permits a modification of the target peptide to introduce a specific functional group, e.g., a C-terminal α-carboxamide group. It may also be desirable to incorporate an endopeptidase cleavage site in the recombinant protein construct, e.g., to facilitate the removal of an N-terminal tail sequence from fragments generated from the recombinant protein construct. Examples of suitable endopeptidases include enterokinase, Factor Xa, ubiquitin cleaving enzyme, thrombin, trypsin, renin, subtilisin, chymotrypsin, clostripain, papain, ficin, and *S. aureus* V8.

Chemical and enzymatic cleavage sites and the corresponding agents used to effect cleavage of a peptide bond close to one of these sites are described in detail in International Application Nos. PCT/US91/04511 and PCT/US94/08125, the disclosure of which is herein incorporated by reference. Examples of peptide sequences (and DNA gene sequences coding therefor) suitable for use as cleavage sites in the

present invention and their corresponding cleavage enzymes or chemical cleavage conditions are shown in Table 1 below. The gene sequence indicated is one possibility coding for the corresponding peptide sequence. Other DNA sequences may be constructed to code for the same peptide sequence.

Table 1

Enzymes for Cleavage	Peptide Sequence	DNA Sequence
Enterokinase	$(Asp)_4Lys$ (SEQ ID NO:14)	GACGACGACGATAAA (SEQ ID NO:13)
Factor Xa	IleGluGlyArg (SEQ ID NO:16)	ATTGAAGGAAGA (SEQ ID NO:15)
Thrombin	GlyProArg or GlyAlaArg	GGACCAAGA or GGAGCGAGA
Ubiquitin Cleaving Enzyme	ArgGlyGly	AGAGGAGGA
Renin	HisProPheHisLeu-LeuValTyr (SEQ ID NO:18)	CATCCTTTTCATC-TGCTGGTTTAT (SEQ ID NO:17)
Trypsin	Lys or Arg	AAA OR CGT
Chymotrypsin	Phe or Tyr or Trp	TTT or TAT or TGG
Clostripain	Arg	CGT
S. aureus V8	Glu	GAA

Chemical Cleavage	Peptide Sequence	DNA Segence
(at pH3)	AspGly or AspPro	GATGGA or GATCCA
(Hydroxylamine)	AsnGly	AATCCA
(CNBr)	Methionine	ATG
BNPS-skatole	Trp	TGG
2-Nitro-5-thiocyanatobenzoate	Cys	TGT

The present method is particularly useful for producing amidated forms of peptides which lack an unblocked cysteine residue, i.e., lack a cysteine residue having a free sulfhydryl group (-SH). Examples of blocked cysteine residues include cystine residues where the sulfur atom is part of a disulfide group (-SS-) and derivatives of cysteine where the hydrogen of the sulfhydryl group has been replaced by a protecting group, e.g., an S-benzylated cysteine residue.

Examples of peptides which lack an unblocked cysteine residue include peptides free of unblocked cysteine residues having a molecular weight of 300 to about 200,000, preferably 400 to 10,000. Such peptides typically include 3 to 100 amino acids residues, preferably 3 to 70 residues. Examples of such peptides include adrenocorticotropic hormone (ACTH), parathyroid hormone (PTH), enkephalins, endorphins, various opioid peptides, β-melanocyte stimulating hormone, glucose-dependent insulinotropic polypeptide (GIP), glucagon, glucagon-like peptides (GLP-I and II), growth hormone-releasing factor (GRF), motilin, thymopoietins, thymosins, ubiquitine, serum thymic factor, thymic humoral factor, various quinines, neurotensin, tuftsin and fragments of these peptides.

The present invention may also be used to produce the α-amidated form of peptides having an -S-S- linkage in their structure. These are also included in the desired peptide. Examples of peptides having amide at their C-terminus and/or an -S-S- linkage include gastrin, calcitonin, calcitonin gene associated peptide, cholecystokinin-pancreozymin (CCK-PZ), eledoisin, epithelial growth factor (EGF), tumor growth factor (TGF-α), pancreastatin, insulin, insulin-like growth factors, luteinizing hormone-releasing hormone (LH-RH), mellitin, oxytocin, vasopressins, pancreatic polypeptide, trypsin inhibitor, relaxin, secretin, somatostatins, somatomedins, substance P, neurotensin,

caerulein, thyrotropin-releasing hormone (TRH), vasoactive intestinal polypeptide (VIP), pituitary adenyl cyclase-activating polypeptides (PACAPs), gastnin-releasing peptide (GRP), endotherins, corticotropin-releasing factor (CRF), PTH-related protein, gallanin, peptide YY, neuropeptide Y, pancreastatin, atrial natriuretic peptides and fragments of these peptides.

The target peptides free of unblocked cysteine residues are preferably linked by intraconnecting peptides which include a cysteine residue. If the cysteine residue is directly adjacent the C-terminus of the target peptide, the construct may be cleaved by an aminolysis reaction to provide a first α-amidated peptide. This is achieved by reacting the cysteine residue with an S-cyanylating agent to form an S-derivatized cysteine residue (activation) and reacting the S-derivatized cysteine residue with an amino compound (aminolysis). More preferably, the intraconnecting peptides include a second cleavage site which permits the N-terminal residues of the first α-amidated peptide to be cleaved to produce a desired α-amidated product peptide.

The S-cyanylating agent may include a thiocyanato substituted aromatic compound or a 1-cyano-4-(dialkyl-amino)pyridinium salt. Suitable examples of the thiocyanato substituted aromatic compound include 4-nitro-thiocyanatobenzene compounds such as 2-nitro-5-thiocyanatobenzoic acid and its salts. Suitable examples of the 1-cyano-4-(dialkylamino)pyridinium salt include 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate (DMAP-CN), 1-cyano-4-(N-methyl,N-benzylamino)pyridinium tetrafluoro-borate and 1-cyano-4-(pyrrolidino)-pyridinium tetrafluoroborate.

A wide variety of amino compounds may be employed in the aminolysis reaction for cleaving the N-terminal peptide linkage of the derivatized cysteine residue to

produce an α-amidated peptide. The amino compound may be ammonia or may be a mono- or disubstituted amine (a "substituted amino compound"). Preferably the amino compound is ammonia or a monosubstituted amine.

The substituent(s) on the substituted amine may be (i) C_{1-20} alkyl, C_{3-8} cycloalkyl, or aryl-C_{1-3} alkyl, which may have no substituent or one to three substituent(s) on their carbon atoms, (ii) amino or alkyl substituted amino, or (iii) hydroxyl or C_{1-6} alkoxy group. Examples of C_{1-20} alkyl substituents includes methyl, ethyl, isopropyl, sec-butyl, neopentyl, octyl, dodecanyl and hexadecanyl. Suitable examples of C_{3-8} cycloalkyl substituents include cyclopentyl, cyclohexyl and methylcyclohexyl. Examples of aryl-C_{1-3} alkyl substituents include benzyl, phenethyl, 3-phenylpropyl and (2-naphthyl)methyl. Examples of C_{1-6} alkoxy group substituents include methoxy, ethoxy, isopropoxy, and hexyloxy.

The substituted amino compound may also be an amino acid or a peptide, e.g., a peptide having from two to about 10 amino acids residues. The α-carboxy group of the amino acid may be in the carboxy form or may be present as a carboxamide. The C-terminal amino acid residue of the peptide may be also be present in the α-carboxy form or as an α-carboxamide. Examples of the amino acid include L- or D-isomer of natural amino acids, such as glycine, alanine or arginine, as well as synthetic amino acids.

The amount of S-cyanylation reagent is about 2 to 50 times, preferably about 5 to 10 times the total amount of all thiol groups. The cyanylation reaction is typically carried out at a temperature in the range from about 0 to 80°C, preferably between about 0 and 50°C. Any buffer can be used as a solvent, as long as it does not react with the cyanylating reagent. Examples of such buffers include Tris-HCl buffer, Tris-acetate buffer, phosphate buffer and borate buffer. An organic

solvent may be present, as long as it does not react with the cyanylating reagent.

The cyanylation reaction is normally carried out at a pH of between 1 and 12. Particularly when using NTCB, a pH range of from 7 to 10 is preferred. When using DMAP-CN, a pH range of from 2 to 7 is preferred, to avoid S-S exchange reaction. The reaction mixture may also contain a denaturant such as guanidine hydrochloride or urea. Under the conditions described above, cyanylation typically is complete within about 10-60 minutes, preferably about 15-30 minutes, although longer reaction times may also be employed.

The derivatized cysteine produced by the S-cyanylation reaction is allowed to react with the amino compound under basic conditions. The pH of the solution is typically determined by the base strength of the amino compound. The amino compound is usually present in the aminolysis reaction mixture at a concentration of about 0.01-15M, and preferably about 0.1-3M.

The derivatized cysteine produced by the S-cyanylation reaction may also be allowed to react with hydroxide (e.g., by adding sodium hydroxide) to produce an intermediate peptide having a C-terminal α-carboxylic acid group. The intermediate peptide may be transpeptidated with an amidated amino acid in the presence of an exopeptidase to produce a C-terminal amidated peptide. Alternatively, where the intermediate peptide includes a C-terminal glycine residue, the terminal glycine residue may be decomposed in the presence of a glycine monooxygenase to produce a C-terminal amidated peptide. The intermediate peptide may also be transamidated with a 2-nitrobenzylamine compound in the presence of a carboxypeptidase to replace the C-terminal intermediate peptide residue with a C-terminal (2-nitrobenzyl)amido group. The (2-nitrobenzyl)amido group may then be photochemically decomposed to produce a C-terminal amidated peptide.

The present invention provides a method of producing an amidated peptide from a recombinant construct which includes a target peptide free of methionine residues. Suitable examples of target peptides lacking a methionine residue include GLP1(1-36) (SEQ ID NO:51), GLP1(7-35) (SEQ ID NO:52), GLP1(7-36) (SEQ ID NO:53), and calcitonin. Preferably, the construct includes two or more copies of the target peptide.

The recombinant protein construct may include multiple copies of a methionine-free target peptide flanked on both ends by a methionine. Constructs of this type may be cleaved into precursor peptides by treatment with cyanogen bromide (CNBr). The precursor peptides are capable of being modified in a controlled manner to generate the desired C-terminal α-amidated product peptides. For example, a recombinant protein construct having a methionine residue as the -(CS2)- cleavage site may be treated with cyanogen bromide to produce a precursor peptide having a C-terminal homoserine residue. The precursor peptide with the homoserine residue in its acid form may then be transamidated, e.g., by treatment with a carboxypeptidase in the presence of an α-amidated amino acid to produce an amidated product peptide.

In a preferred version of the invention, the target peptides, which lack a methionine residue, are linked solely by a methionine (i.e., n and m are 0). Treatment of the recombinant protein construct with cyanogen bromide provides a fragment having the formula TargP-Hse. If the TargP-Hse fragment is present as its α-carboxylic acid form, the fragment may be transamidated with a carboxypeptidase, thereby replacing the terminal homoserine residue. The transamidation permits the homoserine residue to be replaced with either an α-amidated amino acid or a 2-nitrobenzylamine compound.

Transamidation of the TargP-Hse fragments in the presence of a carboxypeptidase may be employed to replace the C-terminal homoserine residue with a 2-nitrobenzylamine compound. Examples of suitable 2-nitrobenzylamine compounds include 2-nitrobenzylamine, (2-nitrophenyl)-glycinamide (ONPGA) and 1-(2-nitrophenyl)-ethylamine. The transamidation reaction produces fragments C-terminated in an (2-nitrobenzyl)amido group, e.g., TargP-NH-(2-nitrobenzyl). The (2-nitrobenzyl)amido fragments may be decomposed by irradiation with long wavelength UV light (e.g., λ no longer than about 320 nm) resulting in the replacement of the (2-nitrobenzyl)amido group with an NH_2 group. The transamidation and decomposition procedures are disclosed in Henriksen et al., J.Am.Chem.Soc., 114, 1876 (1992), the disclosure of which is incorporated herein by reference.

Alternatively, the TargP-Hse fragments may be transpeptidated with an α-amidated amino acid in the presence of a carboxypeptidase such as carboxypeptidase Y. For example, the peptide fragment GLP1(1-35)-Hse (SEQ ID NO:54), may be subjected to transpeptidation with Arg-NH_2 in the presence of a suitable carboxypeptidase to produce GLP1(1-36)-NH_2 (SEQ ID NO:51). One example of such a peptidase is described in International Application No. PCT/US95/06682, the disclosure of which is herein incorporated by reference.

In another embodiment of the invention, the target peptide is free of methionine and unblocked cysteine residues. Target peptides of this type may be produced using a recombinant protein construct of the formula:

$$\text{-TargP-(CS2)-[-(Ln1)}_n\text{-(CS1)-TargP-(CS2)-]}_r\text{,}$$

where the -(CS1)- cleavage site is a methionine residue, and the -(CS2)- cleavage site is a cysteine residue ("multicopy construct MC"), i.e., the variable polypeptides are connected by a -Cys-$(Ln1)_n$-Met- linking peptide. This recombinant protein construct may be

cleaved by reacting the methionine residue with cyanogen bromide to produce fragments having a C-terminal homoserine residue. The peptide fragments may be reacted with an S-cyanylating agent to derivatize the cysteine residue. When the S-derivatized cysteine residue is treated with an amino compound, the remains of the linking peptide are cleaved at the N-terminal peptide bond of the derivatized cysteine residue to furnish an α-amidated peptide. Where the amino compound is a substituted amine (e.g., NH_2R where R is alkyl, alkoxy, -OH or $-NH_2$), the aminolysis reaction provides the corresponding substituted carboxamide, hydroxamic acid derivative or hydrazide.

Alternatively, the multicopy construct may be initially treated with the S-cyanylating agent. Cleavage of resulting cysteine-derivatized construct with an amino compound creates peptide fragments having a C-terminal α-amidated residue and the N-terminal tail sequence ITC-$(Ln1)_n$-Met- (where ITC represents the (iminothiazolinyl)-carbonyl residue generated from the reaction of the derivatized cysteine). The fragments may be cleaved at the N-terminal peptide bond of the methionine residue to remove the N-terminal tail sequence and the furnish desired C-terminal α-amidated peptide.

In another version of this embodiment, after treating the multicopy construct with the S-cyanylating agent, the resulting cysteine-derivatized construct may be treated with CNBr to produce peptide fragments having a C-terminal tail sequence -drCys-$(Ln1)_n$-Hse- (where -drCys- represents a derivatized cysteine residue generated from the cyanylation reaction and -Hse- represents a homoserine residue). The fragments may be reacted with the amino compound to cleave the fragments at the N-terminal peptide bond of the -drCys- residue to remove the C-terminal tail sequence and the furnish an α-amidated peptide.

A suitable example of a recombinant protein construct having a target peptide is free of methionine and unblocked cysteine residues is a recombinant protein construct which includes tandemly linked multiple copies of the sequence -Ala-Met-GLP1(7-36)-Cys- (SEQ ID NO:55). This recombinant protein construct may be treated in sequence with CNBr, an S-cyanylating agent (e.g., NTCB or DMAP-CN) and an amino compound (HNRR') to produce the amidated peptide GLP1(7-36)-NRR' (SEQ ID NO:53).

Another embodiment of the invention provides recombinant protein construct which includes an amino acid sequence of the formula:

-Xxx-(CS1)-TargP-(Cys)-

wherein the -Xxx- is an amino acid residue, the -(CS1)- is a cleavage site and the -TargP- is a target peptide. The target peptide and the -(CS1)- cleavage site are free of unblocked cysteine residues. The target peptide is also free of amino acid sequences corresponding to the -(CS1)- cleavage site. If the -(CS1)- cleavage site is a chemical cleavage site, such as Met, Asn-Gly, Asp-Gly, or Asp-Pro, the target peptide can be cut out of the recombinant protein construct without the use of an enzymatic step.

Amidated peptides may also be produced from a target peptide which may include both a methionine residue and a cysteine residue. Target peptides of this type are incorporated into a multicopy construct which includes an endopeptidase cleavage site. The endopeptidase cleavage site is preferably designed so that the construct may be simultaneously cleaved and transpeptidated with the endopeptidase to produce fragments having a C-terminal α-amidated amino acid residue. The transpeptidation reaction is carried out in the presence of an amino acid or peptide having a C-terminal α-carboxamide using an endopeptidase such as trypsin or thrombin. This method is described in detail

in International Application No. PCT/US91/04511, the disclosure of which is herein incorporated by reference.

Methods for expression of single- and multicopy fusion recombinant polypeptide, e.g., a polypeptide expressed with a leader sequence, such as an affinity moiety attached to it, are known in the art and described in Protein Purification: From Mechanisms to Large-Scale Processes, Michael Ladisch, editor; American Chemical Society, publisher (1990), the disclosure of which is incorporated herein by reference. Methods for expression of multicopy protein constructs lacking a leader sequence are also known in the art (see, e.g., Kirshner et al., J. Biotechnology, 12:247-260 (1989), and Shen, Proc.Natl.Acad.Sci., USA, 81, 4627 (1984), the disclosure of which is incorporated herein by reference).

The invention will be further described by reference to the following detailed examples.

Example 1. Description of the Host Cells

The bacterial host for expression, *E. coli* BL21 F⁻ ompT $r_B^- m_B^-$ (DE3) was obtained from Novagen, Inc., Madison, WI. These *E. coli* cells give high levels of expression of genes cloned into expression vectors containing the bacteriophage T7 promoter. Bacteriophage (DE3) which contains the T7 RNA polymerase gene has been integrated into the chromosomal DNA of the BL21 (DE3) cells. The T7 RNA polymerase gene is controlled by the lacUV5 promoter and the lacI gene.

Example 2. Expression Plasmids Containing hCAII

Construction of pBN1

An expression vector, pET31F1mhCAII containing the hCAII gene was obtained from Dr. P.J. Laipis at the University of Florida. The pET31F1mhCAII was prepared as described by Tanhauser et al., Gene, 117, 113 (1992). Plasmid pET31F1mhCAII contains the coding region for

hCAII (human carbonic anhydrase II) downstream of a bacteriophage T7 promoter in a pUC-derived plasmid backbone. Two synthetic oligonucleotides, 5'-A GCT TTC GTT GAC GAC GAC GAT ATC TT-3' (SEQ ID NO:19) and its complementary sequence 5'-AGC TAA GAT ATC GTC GTC GTC AAC GAA-3' (SEQ ID NO:20), were cloned into pET31F1mhCA2 which had been digested with Hind III. This plasmid was designated pA1 (see Table 2).

Plasmid pA1 was digested with the restriction endonucleases Ssp I and BspE I and the resulting ends were made blunt by treatment with T4 DNA polymerase. The DNA fragment from the pA1 digest containing the T7-hCAII-cassette was subcloned into the Sca I restriction site of pBR322 (New England Biolabs) thus conferring tetracycline resistance, but not ampicillin resistance. The resulting plasmid was designated pBN1.

Construction of pBN3

The pA1 plasmid was opened at the Hind III site and the EcoR V site and the synthetic oligonucleotide, 5'-A GCT GAA TTC AAC GTT CTC GAG GAT-3' (SEQ ID NO:21) and its complementary sequence 5'-ATC CTC GAG AAC GTT GAA TTC-3' (SEQ ID NO:22), were cloned into the vector. The insertion of these oligonucleotides provides a T7-hCAII-cassette containing unique EcoR I and Xho I restriction sites at the carboxyl terminal of hCAII. The resulting plasmid was designated pA3.

The pBN1 vector was digested with EcoR I and the single stranded overhangs were filled in with Polymerase I Large (Klenow) Fragment. The linear plasmid with newly formed blunt ends was religated, thus destroying the EcoR I site. The resulting plasmid was designated pBN3.

Plasmid pA3 was digested with the restriction endonucleases, Xba I and BspE I. The DNA fragment from the pA3 digest containing the T7-hCAII-cassette was subcloned into the pBN1 vector which had been digested

with Xba I and BspE I. The resulting vector was designated plasmid pBN4.

Example 3. Expression Plasmid without hCAII

All the nucleotides coding for the hCAII gene were removed from the expression vector pBN1 by the following procedure. Two synthetic DNA strand were synthesized:

Oligo A: 5' AAT CTA GAA ATA ATT TTG TTT AAC TTT AAG AAG G (SEQ ID NO:23)

Oligo B: 5' TAG AAT TCC ATG GTA TAT CTC CTT CTT AAA (SEQ ID NO:24)

Oligonucleotides A and B were used in a PCR amplification of the primer-dimer. The PCR product was purified and digested with the restriction endonucleases EcoR I and Xba I. The resulting fragment was ligated into the vector pBN4, which had previously been digested with EcoR I and Xba I. The resulting vector, designated pBN5, contains unique sites for the restriction endonucleases Nco I and Xho I between the T7 promoter and the T7 terminator. Plasmid pBN5 may be used to form vectors coding for multicopy constructs having at least about 2 copies of a target peptide. The resulting plasmids may be used to transform host cells such as E. coli and express the multicopy constructs as part of inclusion bodies.

Example 4. General Procedure for Preparation of Transformed Cell Lines

Competent *E. coli* cells were purchased from Novagen containing the DE3 bacteriophage. The *E. coli* BL21 (DE3) cells were transformed with the desired plasmid according to Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. (1989). Ampicillin or tetracycline resistant clones (those containing the recombinant plasmid) were selected and subcultured for subsequent screening.

Selection procsdure.

DNA was isolated from subcultured cells by conventional methods (Promega-Wizard Mini Prep Kit). The purified DNA was digested with specific restriction endonucleases to select clones containing the correct plasmids. Purified DNA from representative screened clones was subjected to DNA sequencing to confirm the presence of the gene for the fusion protein construct.

Isolation and prsservation of cell linss.

E. coli cells containing the expression plasmid for the fusion protein were plated on LBT agar and incubated for 12 hours at 37°C. Using sterile conditions, several single cell isolates were transferred to culture flasks containing LBT broth supplemented with glucose at 1 mg/ml media and incubated at 37°C with shaking for 12 to 16 hours.

To each of the culture flasks were added 50 ml of sterile glycerol containing 750 μg tetracycline. The contents of the flasks were thoroughly mixed then 1.0 ml aliquots are transferred to 2 ml cryovials under sterile conditions. The cryovials were cooled to -20°C for 30 minutes, then transferred to a liquid nitrogen dewar and maintained at -176°C. The frozen vials of culture were used to prepare the inoculum.

Example 5. General Procedure for Fermentation and Isolation of Inclusion Bodies

Preparation of the inoculum.

L-broth was sterilized in the autoclave at 121°C for 20 minutes on the liquid cycle setting. The glucose and tetracycline stocks were filter sterilized by passage of the solution through a 0.22 μm filter. Two 250 ml shake flasks were each charged with the following solutions:

50 ml L-broth (1.0 % tryptone, 1.0% NaCl, 0.5% yeast extract)

1.0 ml glucose stock (50 mg/ml)

150 μl tetracycline stock (5.0 mg/ml)

100 µl thawed inoculum of *E. coli* cells transformed with a vector coding for the desired hCA-fusion construct

The shake flasks were placed in an incubator shaker at 37°C, 200-220 rpm for 10-14 hours. The optical density (O.D.) of the cells in the resulting solutions was then measured at 540 nm. A 1:25 dilution was usually necessary to obtain a proper reading. One of the two shake flasks was then chosen for inoculating the next set of shake flasks. Three 500 ml shake flasks were each charged with the following sterilized solutions:

200 ml L-broth (1.0 % tryptone, 1.0% NaCl, 0.5% yeast extract);

4.0 ml glucose stock (50 mg/ml);

600 µl tetracycline stock (5.0 mg/ml);

1.0 ml inoculum from one of the first two shake flasks.

The three shake flasks were placed in an incubator shaker under the conditions described above and the cells were allowed to grow for 8-10 hours. The optical density of the resulting solutions was then measured at 540 nm (typically at a 1:25 dilution). All three shake flasks were then used to inoculate the fermentor.

Fermentation

Fermentation media was added to the fermentor and the volume was adjusted to 45.0 L with distilled H_2O. The media contained the following: 1200.0 g Casamino acids; 300.0 g Yeast extract; 30.0 g NaCl; and 0.10 ml Antifoam. The fermentor was sterilized at 121°C for 25 minutes. The fermentor was cooled to 37°C. Before inoculation, the following solutions were added to the fermentor:

glucose (480.0 g in 800.0 ml H_2O)

magnesium (120.0 g $MgSO_4 \cdot H_2O$ in 250.0 ml H_2O)

phosphates (120.0 g K_2HPO_4 & 465.0 g KH_2PO4 in 3.0 l H_2O)

tetracycline (0.90 g tetracycline·HCl in 30.0 ml 95% EtOH & 20.0 ml H_2O)

mineral mix (Dissolved in 490.0 ml H_2O & 10.0 ml concentrated HCl):

3.6 g $FeSO_4 \cdot 7H_2O$
3.6 g $CaCl_2 \cdot 2H_2O$
0.90 g $MnSO_4$
0.90 g $AlCl_3 \cdot 6H_2O$
0.09 g $CuCl_2 \cdot 2H_2O$
0.18 g Molybdic Acid
0.36 g $CoCl_2 \cdot 6H_2O$

All of the above solutions were sterilized for 20 minutes in the liquid cycle in an autoclave except for the tetracycline and mineral mix solutions. These were sterilized by passage through a 0.22 μm filter. At this point, the pH typically had dropped to approximately 6.5. If this had occurred, base (14.8 N ammonium hydroxide) was added to adjust the pH to 6.8. After the pH reached 6.8, 600.0 ml inoculum was added to the fermentor. The following parameters were monitored at time zero and throughout the fermentation: Glucose concentration (maintained at about 2-5 g/l); Optical Density; pH (6.8 is optimal); Dissolved Oxygen (40% is optimal); and Agitation. The temperature was maintained at 37°C throughout the fermentation. Air intake was 40 l/min at the beginning of the fermentation. The initial dissolved oxygen concentration was 90% but quickly dropped to 40%. It was maintained at this level via increased agitation and oxygen supplementation throughout the fermentation. When oxygen supplementation was started, the air influx was reduced to 20 l/min. The initial glucose concentration was approximately 9 g/l but dropped to 5 g/l after about six hours. Once the glucose concentration dropped to this level, a glucose feed (70% w/v glucose) was used to maintain the glucose concentration at 5 g/l.

When the fermentation had proceeded to the point where an O.D. of 15-20 was measured, the media feed was started. The media feed consisted of 1200.0 g casamino acids and 300.0 g yeast extract dissolved in 5.0 l distilled H_2O and autoclaved for 20 minutes on liquid cycle. The media feed was added to the fermentor over 1.0-1.5 hours. When fermentation had produced an O.D. of 30.0, the fermentation was induced by adding the following solutions to the fermentor: isopropylthiogalactoside (IPTG; 28.8 g in 200 ml distilled H_2O); $ZnCl_2$ (0.818g in 50 ml distilled H_2O with one drop of 6N HCl). The IPTG solution was filter sterilized through 0.22 μm filter. The $ZnCl_2$ solution was sterilized for 20 minutes, liquid cycle in the autoclave. After the addition, the concentration of IPTG in fermentor was 2.0 mM and the concentration of $ZnCl_2$ was 100 μM. A feed of a mixture of amino acids was then started at this point. The amino acid feed consisted of 225.0 g L-serine; 75.0 g L-tyrosine; 74.0 g L-tryptophan; 75.0 g L-phenylalanine; 75.0 g L-proline; and 75.0 g L-histidine; and was dissolved in a mixture of 1.5 l H_2O and 500 ml concentrated HCl. The amino acid feed was sterile filtered through a 0.22 μm filter prior to addition to the fermentation. Induction was allowed to continue for 2.0 hours at which point the fermentation broth was transferred to a harvest tank and chilled to approximately 5-10°C. The fermentation typically yielded between 6.5 to 9.5 kg of wet cell paste (dry cell weight of about 1.0-1.5 kg).

Cell Harvest

The cell suspension from the fermentor as desribed above was concentrated over a tangential crossflow membrane to a volume of 10 l. The concentrated cell suspension was diafiltered and washed with 30 l of a cold wash buffer containing 50 mM Tris-SO4 pH 7.8, 1.0 mM EDTA, and 0.10 mM phenylmethylsulfonyl fluoride (PMSF). The cell suspension was then concentrated to 8

1. At this point, the concentrated cell suspension (cell paste) may be bagged and frozen for later processing or transferred to homogenizer holding tanks for cell lysis.

Cell Lysis

The cell paste obtained from the 60 l fermentor was diluted to 32 l in cold wash buffer (see above) and the resulting cell suspension was chilled to 5-10°C. The chilled cell suspension was homogenized at 12,000 psi with a Galin high pressure homogenizer. The homogenized cell paste was passed through a heat exchanger to chill the lysate to 10°C and passed through the homogenizer a second time.

__Example 6. Expression Plasmid for PTH Single Copy__

The preparation of the DNA segment coding for a single copy PTH(1-34) fusion protein was carried out by preparing an expression vector coding for a PTH-construct which included a DNA segment coding for the hCAII, an interlinking peptide, and PTH(1-34). The following oligonucleotides were obtained from Operon Technologies Inc, 1000 Atlantic Ave. Alameda CA 94501.

Oligo 1: 5' CCC AAG CTT CTG TTC GTG GTC CGC GTT CTG TTT CTG AAA (SEQ ID NO:25)

Oligo 2: 5' GAA ACA GAA CGC GGA CCA CGA ACA GAA GCT TGG G (SEQ ID NO:26)

Oligo 3: 5' TCC AGC TGA TGC ACA ACC TGG GTA AAC ACC TGA ACT (SEQ ID NO:27)

Oligo 4: 5' AGG TGT TTA CCC AGG TTG TGC ATC AGC TGG ATT TCA (SEQ ID NO:28)

Oligo 5: 5' CTA TGG AAC GTG TTG AAT GGC TGC GTA AAA AAC TGC A (SEQ ID NO:29)

Oligo 6: 5' TTT TTT ACG CAG CCA TTC AAC ACG TTC CAT AGA GTT C (SEQ ID NO:30)

Oligo 7: 5' GGA CGT TCA CAA CTT CTA AGA TAT CCG G (SEQ ID NO:31)

Oligo 8: 5' CCG GAT ATC TTA GAA GTT GTG AAC GTC CTG CAG (SEQ ID NO:32)

The eight synthetic DNA oligonucleotides were obtained and the complementary strands were phosphorylated and annealed. The four double stranded fragments were used to prepare a DNA fragment coding for the interpeptide linker followed by PTH(1-34) (SEQ ID NO:56). This DNA fragment was digested and inserted into pA1 using the restriction sites Hind III and EcoR V creating the vector pA1:PTH(1-34).

The expression cassette from pA1:PTH(1-34) was transferred to pBN1 (described above) by digesting both vectors with Xba I and BspE I and then ligating the aproximately 1100 base pair DNA fragment from pA1:PTH(1-34) containing the T7 expression cassette into the digested pBN1. The resulting plasmid was designated pBN1:PTH(1-34).

Example 7. Expression Plasmid for PTH Single Copy with a Cyanylation Site

The following two DNA oligonucleotides are synthesized:

Oligo C: 5' TC AAA GCT TCT GCC ATG GGC GGC CGC GTC GAC CGT GGT CCG CGT TCT GTT TCT GAA ATC CAG (SEQ ID NO:33)

Oligo D: 5' CTC GAT ATC TTA CTC GAG AGC GCA GAA GTT GTG AAC GTC C (SEQ ID NO:34)

Oligonucleotide C includes Hind III, Nco I, Not I and Sal I sites positioned in front of a thrombin-cleavable linking peptide. Oligonucleotide D inserts a cysteine immediately after the PTH(1-34) (SEQ ID NO:56) followed by a a Xho I site, a stop codon and the EcoR V site.

The plasmid pBN1:PTH(1-34) is used as a template and the Oligonucleotides C and D are used as primers for the PCR amplification of the single copy gene.

The PCR-product and plasmid pA1 are digested with the restriction endonucleases Hind III and EcoR V and the PCR-product is ligated into the vector. The resulting plasmid is designated pA1:PTH(1-34)C-1C. The

T7 expression casette from pA1:PTH(1-34)C-1C is transferred to pBN1 by digesting both vectors with Xba I and BspE I, and subsequently ligating the approximately 1100 base pair fragment containing the expression casette from pA1:PTH(1-34)C-1C into the pBN1. The resulting plasmid is designated pBN1:PTH(1-34)C-1C. Figure 1 depicts a portion a the DNA sequence (and the corresponding peptide sequence) of pBN1:PTH(1-34)C-1C.

<u>Example 8. Expression Plasmids for PTH Multicopies</u>

Double Copy construct:

The plasmid pBN1:PTH(1-34)C-1C is digested with Sal I and BspE I and the fragment containing the DNA sequence coding for the thrombin site, the desired polypeptide, and the T7-terminator is purified. The fragment is then inserted into the plasmid pBN1:PTH(1-34)C-1C, which has been digested with Xho I and BspE I. The resulting plasmid is designated pBN1:PTH(1-34)C-2C. Figure 2 shows a portion a the DNA sequence (and the corresponding peptide sequence) of pBN1:PTH(1-34)C-2C.

Four Copy Construct:

The plasmid pBN1:PTH(1-34)C-2C is digested with Sal I and BspE I and the DNA sequence containing the thrombin site through the T7-terminator is purified. The fragment is then inserted into pBN1:PTH(1-34)C-2C which had been digested with Xho I and BspE I to yield pBN1:PTH(1-34)C-4C. Plasmids having higher numbers of copies of the PTH(1-34) sequence (SEQ ID NO:56) may be made using the same strategy.

<u>Example 9. Expression Plasmid for PTH Multicopy without hCAII</u>

Multicopy constructs having at least 2 copies of the PTH(1-34) sequence (SEQ ID NO:56) may be expressed as inclusion bodies from a construct which does not contain hCAII sequence. For example, constructs including at least 2 copies of the PTH(1-34) sequence (SEQ ID NO:56) may be expressed.

The plasmid pBN1:PTH(1-34)C-x_c (where x represents the number of copies of the PTH(1-34) sequence present in the plasmid) may be digested with Nco I and BspE I and ligated into the expression vector pBN5, which has also been digested with the same restriction endonucleases, to provide the plasmid pBN5:PTH(1-34)C-x_c.

Higher copy numbers may be made by digestion of plasmid pBN5:PTH(1-34)C-y_c (where y represents the number of copies of the PTH(1-34) sequence present in the plasmid) with Sal I and BspE I, purifying the DNA fragment containing the multicopy gene and inserting the fragment into the plasmid pBN5:PTH(1-34)C-x_c which had been digested with Xho I and BspE I. The resulting fragment is designated: pBN5:PTH(1-34)C-$(x+y)_c$, where x+y represents the number of copies of the PTH(1-34) sequence present in the plasmid.

<u>Example 10. Production of PTH(1-34)-NH_2</u>

Competent *E. coli* BL21 F^- ompT r_B^- m_B^- (DE3) host cells may be transformed with plasmid pBN1:PTH(1-34)C-2C and cultured according to the procedures described in Examples 4 and 5 above. A portion of the nucleotide sequence which encodes two copies of PTH(1-34) and flanking sequences is shown in Figure 2.

The transformed cells may be lysed and the inclusion bodies containing the hCA-PTH fusion protein construct isolated by centrifugation. The inclusion body pellet obtained from centrifugation is dissolved in 50mM NaOH, and the pH was immediately reduced to 8.1 by the addition of 1M Tris-HCl. Thrombin is added (thrombin/construct weight ratio of 1 to 1500) and proteolysis is allowed to occur for 48 hours at 37°C. The reaction is terminated by rendering the solution 0.1 mM with respect to PMSF. In addition to cleaving the fragment containing hCAII (hCAII fragment) from the remainder of the construct, the thrombin treatment cleaves the multicopy portion of the construct into two

pre-PTH fragments. Each pre-PTH fragment contains a single copy of PTH(1-34) flanked at the C-terminus by a cysteine residue (i.e., PTH(1-34)-Cys-Ttt where Ttt is a C-terminal tail sequence). The hCAII fragment is precipitated by rendering the solution 90 mM with respect to citric acid and the precipitate is removed by centrifugation. The pre-PTH fragments remain in the supernatant fluid.

Cyanylation/Amidation of the Pre-PTH Fragments

After desalting the supernatant by a low pressure C8 column, the pre-PTH fragments may be dissolved in a pH 3.5 urea/ammonium acetate buffer and treated with excess DTT and DMAP-CN at room temperature for 15-30 minutes. The reaction mixture is then immediately desalted, e.g., using an low pressure C8 column. The desalted S-derivatized fragments are then dissolved in 3M aqueous ammonia and allowed to react for 30 minutes at 0°C to produce recombinant PTH(1-34)-NH_2. The PTH(1-34)-NH_2 may be further purified by HPLC on a semi-preparative C18 column using an acetonitrile gradient.

Example 11. Expression Plasmids for GRF Single Copy

A GRF-construct was made consisting of a DNA segment coding for the hCAII, an interlinking peptide and GRF(1-44). An oligonucleotide coding for an interlinking peptide sequence (FVNGPRAMVDDDDK (SEQ ID NO:35)) was substituted for the last three residues of hCAII, SFK. The double-stranded oligonucleotide for the product peptide sequence was inserted directly after the peptide linker region. The gene sequence of the interlinking peptide region coded for a series of amino acids with unique sites that can either be processed chemically or by proteases to release the desired product peptide.

Oligonucleotides corresponding to segments of the linker and the peptide were obtained from the DNA

Synthesis Core Facilities of the Interdisciplinary Center for Biotechnology at the University of Florida.

Modification of the hCAII sequence.

Eight oligonucleotides containing segments of the linker and the peptide were phosphorylated and complimentary oligonucleotide pairs 1&2, 3&4, 5&6, and 7&8 were annealed. Oligonucleotide pairs 1&2 and 3&4 were simultaneously joined into the pTZ19R vector (commercially available from Pharmacia Biotech Inc., NJ) between the Hind III and Sal I sites to yield pTZ:GRF(1-29). Oligonucleotide pairs 1&2 and 3&4 were simultaneously ligated into a separate pTZ19R vector between the Sal I and EcoR I sites to yield pTZ:GRF(29-44)A. The gene fragments were cloned adjacent to each other in a single vector by digesting pTZ:GRF(1-29) and pTZ:GRF(29-44)A with the restriction endonucleases Xmn I and Sal I, isolating the 1.9kb band from the pTZ:GRF(1-29) vector and the 0.9 kb band from the pTZ:GRF(29-44)A and ligating them together to yield the vector pTZ:GRF(1-44)A.

The three Asn-Gly sites in hCAII (located at positions 10-11, 61-62, and 230-231) were changed to Gln10-Gly11, Gln61-Gly62 and Asn230-Ala231. The changes were made by site directed mutagenesis of specific codons in pA1. Oligonucleotide containing the desired mutations for Asn61-Gly62 along with another primer to the carboxy end of hCAII were used to amplify the portion of the gene containing the sequence to be changed. The PCR fragment was digested with Hind III and BamH I and ligated into pA1 at the restriction sites mentioned above.

The Asn10-Gly11 and Asn230-Gly231 mutations were created using Amersham's Site Directed Mutagenesis Kit. The three oligonucleotide sequences are given below:

positions 10-11: 5' GGC AAA CAC CAG GGA CCT GAG CAC TG (SEQ ID NO:36)

positions 61-62: 5' CTG AGG ATC CTC AAC CAG GGT CAT GCT TTC (SEQ ID NO:37)

positions 230-231: 5' CTT AAC TTC AAT GCG GAG GGT GAA CC (SEQ ID NO:38)

All three mutations were combined to create the plasmid pA2. The pA2 vector was digested with EcoR V. pTZ:GRF was digested with Dra I and EcoR V. The fragment containing the GRF gene and the linearized pA2 plasmid were ligated together to yield pA2:GRF(1-44)A. Both pA2:GRF(1-44)A and pBN2 were digested with Xba I and BspE I. The expression cassette from the pA2:GRF(1-44)A was ligated into pBN1 to yield the final expression vector pBN2:GRF(1-44)A.

Example 12. Expression Plasmids for GRF Multicopies

Plasmids containing nucleotide sequence coding for multiple copies of GRF fused to hCAII were prepared. The constructs contain an interlinking peptide between the individual copies of GRF. The interlinking peptide includes a cysteine residue and an enterokinase cleavage site. In front of the first copy of the peptide and downstream of the hCAII gene a linker which contains a thrombin cleavage site, a cysteine cleavage site and an enterokinase site, is inserted. This provides more flexibility in the purification of the product peptide.

A portion of the resulting construct is shown in Figure 3.

Construction af Single Copy Gene.

Six oligonucleotides were obtained from GENE LINK INC., 401 Clairmont Ave, Thornwood NY 10594.

Oligo 1: 5' TGC TGC AGG ACA TCA TGT CCC GTC AGC AGG GTG AAT CTA AC (SEQ ID NO:39)

Oligo 2: 5' CCG AAT TCG ATA TCT TAC TCG AGC ATA GCG CAC AGA CGA GCA CGA GCA CC (SEQ ID NO:40)

Oligo 3: 5' CAA AGC TTT CGC CAT GGT CGA CGA CGA CGA CAA ATA CGC TGA CGC TAT CTT CAC CAA CTC T (SEQ ID NO:41)

Oligo 4: 5' GTC CTG CAG CAG TTT ACG AGC AGA CAG CTG ACC CAG AAC TTT ACG GTA AGA GTT GGT GAA (SEQ ID NO:42)

Oligo 5: 5' CCA AAG CTT TCG GTG GTG GTG GTG GTC CGC GTG GT (SEQ ID NO:43)

Oligo 6: 5' GTC GTC GAC CAT GGC GCA ACC ACG CGG ACC (SEQ ID NO:44)

A cysteine was inserted between the codon for the last amino acid in GRF and the stop codon. Additional restriction endonuclease sites Xho I, EcoR V and EcoR I site were inserted to ease the construction of the multicopy construct.

The last part of the gene construct was PCR-amplified using oligonucleotides 1 and 2 as primers and pBN2:GRF(1-44)A as a template. The PCR product and the vector pUC19 (commercially available form NEW ENGLAND BIOLABS, Inc., 32 Tozer Road, Beverly MA 01915-5599) were digested with the restriction endonucleases Pst I and EcoR I. The PCR product was then ligated into the digested vector to yield pUC19:GRF(22-44)C.

The middle part of the gene construct was PCR-amplified where oligonucleotides 3 and 4 are overlapping primers that were filled in by the Taq polymerase during the thermocycling process. The PCR product and the vector pUC19:GRF(22-44)C were digested with the restriction endonucleases Pst I and Hind III. The PCR product was then ligated into the digested vector to yield pUC19:GRF(1-44)C.

The gene sequence for the interlinking peptide was modified as follows. The front part of the gene construct was PCR-amplified where oligonucleotides 5 and 6 are overlapping primers that are filled in by the Taq polymerase. The PCR product and the vector pUC19:GRF(1-44)C were digested with the restriction endonucleases

Hind III and Sal I. The PCR product was then ligated into the digested vector to yield pUC19:GRF(1-44)C-1_c.

The gene sequence for the interlinking peptide and GRF construct was transferred from pUC19:GRF(1-44)C-1_c to pA2 as follows. Plasmids pUC19:GRF(1-44)C-1_c and pA2 were digested with Hind III and EcoR V and the DNA sequence for the interlinking peptide and the desired gene construct was purified and ligated into the pA2, which also was digested with the same restriction endonucleases. This yielded the vector pA2:GRF(1-44)C-1_c. This plasmid may be used for expression with ampicillin resistance.

The expression cassette of the pA2:GRF(1-44)C-1_c was transferred to pBN1 by digestion of both pA2:GRF(1-44)C-1_c and pBN1 with the restriction endonucleases Xba I and BspE I. The segment for the fusion protein was ligated into the pBN1 to yield the final expression vector pBN2:GRF(1-44)C-1_c (see Figure 3 which depicts a portion of the plasmid).

Double Copy Construct.

The plasmid pBN2:GRF(1-44)C-1_c may be digested with Sal I and BspE I and the DNA fragment containing the enterokinase site, the GRF(1-44)C and the T7-terminator DNA sequence is purified. The fragment is then inserted into pBN2:GRF(1-44)C-1_c which has been digested with Xho I and BspE I to yield pBN2:GRF(1-44)C-2_c (see Figure 4).

Four Copy Construct.

The plasmid pBN2:GRF(1-44)C-2_c may be digested with Sal I and BspE I and the DNA fragment containing the sequence coding for the enterokinase site, the desired peptide and the T7-terminator is purified. The fragment is then inserted into pBN2:GRF(1-44)C-2_c which has been digested with Xho I and BspE I to yield pBN2:GRF(1-44)C-4_c.

Example 13. Expression Plasmid for GRF Multicopy without hCAII

The plasmid pBN2:GRF(1-44)C-x_c (where x represents the number of copies of the GRF sequence present in the plasmid) may be digested with Nco I and BspE I and ligated into the expression vector pBN5, which has also been digested with the same restriction endonucleases yielding the vector pBN5:GRF(1-44)C-x_c.

The number of copies of GRF(1-44) (SEQ ID NO: 57) in a plasmid may be increased by digestion of plasmid pBN5:GRF(1-44)C-y_c (where y represents the number of copies of the GRF sequence present in the plasmid) with Sal I and BspE I, purifying the DNA fragment containing the multicopy gene and inserting it into the plasmid pBN5:GRF(1-44)C-y_c, which had been digested with Xho I and BspE I, and ligating the purified fragments. The resulting plasmid is designated pBN5:GRF(1-44)C-$(x+y)_c$, where x+y represents the number of copies of the GRF sequence present in the plasmid.

Example 14. Production of GRF(1-44)-NH_2 from a Multicopy Construct

Competent *E. coli* BL21 F^- ompT r_B^- m_B^- (DE3) host cells may be transformed with the plasmid pBN2:GRF(1-44)C-4C and cultured according to the procedures described in Examples 4 and 5 above. The cells are lysed and the inclusion bodies containing the hCA-GRF fusion protein construct are isolated by centrifugation.

The frozen pellet from above (10 to 20 g) containing the fusion protein is added to 2 l of 50 mM NaOH containing 0.25 g N-lauryl-sarcosine. After being homogenized to insure complete dissolution, the pH is confirmed to be between 11.6 and 11.9. The solution is sonicated to insure that the last trace of pellet dissolved. At this point the protein concentration is between 12 and 15 mg/ml. The pH of the solution is then adjusted to 8.0 to 8.2 with 1 M Tris-HCl and the resulting solution filtered through a 0.45 μm membrane.

Thrombin may be added to the peptide at a weight ratio of 1 to 15,000, respectively. Proteolysis of the interlinking peptide is allowed to proceed at 37°C for 22 to 24 hours. The reaction is terminated by rendering the solution 0.1 mM with respect to PMSF. The resulting solution may be used immediately or stored at -80°C.

The thrombin digested fusion protein is rendered 90 mM with respect to citric acid thereby causing the N-terminal fragment containing the hCAII peptide to precipitate. The protein precipitate may be removed by centrifugation. The supernatant containing the multicopy GRF fragment is filtered through a 0.45 μm filter.

The multicopy GRF fragment may be dissolved in a pH 3.5 urea/ammonium acetate buffer and treated with excess DMAP-CN (an S-cyanylating agent) at room temperature for 15-30 minutes. The reaction mixture is then immediately desalted, e.g., using an low pressure C8 column. The desalted, S-derivatized multicopy fragment may be dissolved in 3M aqueous ammonia and allowed to react for 30 minutes at room temperature to produce pre-GRF fragments. The pre-GRF fragments include the amino acid sequence DDDDK-GRF(1-44)-NH_2 (SEQ ID NO:58).

The solution of the pre-GRF fragments is diluted with H_2O to produce a peptide concentration of 1.0 mg/ml. Triton X-100 is added to a final concentration of 0.1%. Succinic acid and calcium chloride are added to produce concentrations of 50 mM (5.9 mg/ml) and 2 mM (0.3 mg/ml) respectively and the solution pH is adjusted to 5.5. After the solution is filtered through a 0.45 μm membrane, 5.0 mg/ml Dowex 1 resin is added. A 1:3000 ratio of enterokinase enzyme is added and the reaction is maintained in a 35-40 °C water bath with constant stirring. After 20-24 hours the cleavage reaction which converts the pre-GRF fragments into GRF(1-44)-NH2 (SEQ ID NO:57) reaches 70-80% completion. The reaction mixture is filtered to remove the Dowex 1 and the

reaction is stopped by the addition of acetonitrile to a final concentration of 15%. The sample may be stored at -80°C. If desired, purification of the GRF(1-44)-NH_2 product may be carried out by preparative HPLC using a C8 column.

Example 15. Expression Plasmid for GLP1(7-36) Multicopy Single Copy Construct.

A GLP1(7-36)-construct was made consisting of a DNA segment coding for hCAII, an interlinking peptide and GLP1(7-36)-Cys-Ala. The interlinking peptide included 5 glycine residues, a thrombin site (Gly-Pro-Arg), a cysteine residue and a cyanogen bromide cleavage site (in order running from the N-terminal to C-terminal). The permits more flexibility in the purification of the construct.

Four oligonucleotides were obtained from GENE LINK INC., 401 Clairmont Ave, Thornwood NY 10594.

Oligo 1: 5′ GTC AAA TTT GGC GGC CGC GGT GGT GGT GGT GGT GTT AAC GGT CCG CGT GGT (SEQ ID NO:45)

Oligo 2: 5′ GTC CTC GAG GGT ACC TTC AGC ATG CAT GTC GAC AGC GCA ACC ACG CGG ACC G (SEQ ID NO:46)

Oligo 3: 5′ CTG GGT ACC TTC ACC TCC GAC GTT TCC TCC TAC CTG GAA GGT CAG GCT GCT AAA GAA TTC (SEQ ID NO:47)

Oligo 4: 5′ CCT GGT CGA CTT ACT CGA GAG CGC AAC GAC CTT TAA CCA GCC AAG CGA TGA ATT CTT TAG C (SEQ ID NO:48)

Oligonucleotides 1 and 2 are overlapping primers which were filled in with Taq polymerase during PCR amplification. The PCR product was digested with the restriction endonucleases Apo I and Xho I and inserted into the pBN4, which had been digested with EcoR I and Xho I. The resulting construct was designated pBN4:GLP(7-11)

Oligonucleotides 3 and 4 are also overlapping primers which were filled in with Taq polymerase during

PCR amplification and the product was digested with Kpn I and Sal I, and ligated into pUC19, which also had been digested with Kpn I and Sal I yielding the construct pUC19:GLP(11-36)C. The pUC19:GLP(11-36)C was digested with Kpn I and Hinc II and inserted into a pA5 vector digested with Kpn I and EcoR V. The resulting vector pA5:GLP(11-36)C was digested with Kpn I and BspE I and the C-terminal backbone of the GLP construct followed by the T7 terminator was transferred into the pBN4:GLP(7-11) digested with Kpn I and BspE I which already contained the hCAII, the interlinking peptide sequence and the GLP(7-11) gene. The final vector was named pBN4:GLP(7-36)C-1_c (see Figure 5) and could be used for production of a single copy GLP fusion construct.

Double Copy Construct.

The plasmid pBN4:GLP(7-36)C-1_c may be digested with Sal I and BspE I and the DNA fragment containing the sequence for the cyanogen bromide site, the desired peptide and the T7-terminator is purified. The fragment is then inserted into pBN4:GLP(7-36)C-1_c, which has been digested with Xho I and BspE I to yield pBN4:GLP(7-36)C-2_c (see Figure 6).

Four Copy Construct.

The plasmid pBN4:GLP(7-36)C-2_c may be digested with Sal I and BspE I and the DNA fragment containing the sequence for the cyanogen bromide site, the desired peptide and the T7-terminator is purified. The fragment is then inserted into pBN4:GLP(7-36)C-2_c, which has been digested with Xho I and BspE I to yield pBN4:GLP(7-36)C-4_c.

Higher Copy Constructs.

Plasmids having a greater number of copies of GLP(7-36) may be prepared by digesting plasmid pBN4:GLP(7-36)C-x_c (where x is the number of copies of GLP in the plasmid) with Sal I and BspE I. The DNA sequence which includes the cyanogen bromide site, the desired peptide and the T7-terminator is purified. The

purified fragment is then inserted into plasmid pBN4:GLP(7-36)C-y_c (where y is the number of copies of GLP in the plasmid), which has been digested with Xho I and BspE I to yield plasmid pBN4:GLP(7-36)C-$(x+y)_c$ (where x+y is the number of copies of GLP in the plasmid). For example, plasmid pBN4:GLP(7-36)C-8_c may be prepared using this method from pBN4:GLP(7-36)C-4_c (i.e, where x and y are both 4).

Example 16. Production of GLP1(7-36)-NH_2

Competent *E. coli* BL21 F^- ompT r_B^- m_B^- (DE3) host cells may be transformed with plasmid pBN4:GLP(7-36)C-2C and cultured according to the procedures described in Examples 4 and 5 above. The cells are lysed and the inclusion bodies containing the hCA-GLP fusion protein construct are isolated by centrifugation.

The inclusion body pellet obtained from the centrifugation is suspended at a concentration of 90 g pellet per l in a buffer containing 2% N-lauryl sarcosine 25 mM Tris HCl, 50 mM EDTA, pH 7.6. The suspension is sonicated in 1 l aliquots for 4 minutes at room temperature.

The solution is centrifuged at 23,400 x g for 10 minutes in Sorvall GSA rotor. The supernatant fluid is made 25% saturated ammonium sulfate by addition of solid, and after 3 hours at 4°C, the precipitate that formed is collected by centrifugation at 23,400 x g for 10 minutes.

The pellets may be resuspended in 50% ethanol (2 l), then centrifuged at 23,400 x g for 10 minutes to collect the pellet. This wash step is repeated once more with 2 l of 50% ethanol. The pellets are then suspended in the centrifuge bottles with 200 ml of 100 mM EDTA per bottle. After sitting for 10 mins, the suspensions are centrifuged at 15,000 x g. This step is repeated once more with distilled H_2O in place of 100 mM

EDTA. The resulting pellets were immediately used in the next step or stored frozen at -80°C.

The frozen pellet from above (10 to 20 g) containing the fusion protein is added to 2 l of 50 mM NaOH containing 0.25 g N-lauryl-sarcosine. After being homogenized to insure complete dissolution, the pH is confirmed to be between 11.6 and 11.9. The solution is sonicated to insure that the last trace of pellet dissolved. At this point the protein concentration is between 12 and 15 mg/ml. The pH of the solution is then adjusted to 8.0 to 8.2 with 1 M Tris-HCl and the resulting solution filtered through a 0.45 μm membrane.

Thrombin may be added to the peptide at a weight ratio of 1 to 15,000, respectively. Proteolysis of the interlinking peptide is allowed to proceed at 37°C for 22 to 24 hours. The reaction is terminated by rendering the solution 0.1 mM with respect to PMSF. The resulting solution may be used immediately or stored at -80°C.

The thrombin digested fusion protein is rendered 90 mM with respect to citric acid thereby causing the N-terminal fragment containing the hCAII peptide to precipitate. The protein precipitate may be removed by centrifugation at 20,000 x g and resuspended in 90 mM Na citrate, pH 3.0. The suspension is centrifuged again, and the supernatant fluid is combined with the first supernatant fluid. The combined supernatants containing the multicopy GLP fragment are filtered through a 0.45 μm filter and stored at -80°C until used. The multicopy GLP fragment released from the fusion protein by the thrombin cleavage includes a 6 amino acid N-terminal tail sequence (Gly Cys Ala Val Asp Met) (SEQ ID NO:59). The two GLP1(7-36) sequences (SEQ ID NO:53) are flanked by an N-terminal methionine residue and a C-terminal Cys-Ala sequence.

The multicopy GLP fragment is absorbed onto a preparative C8 reverse phase column equilibrated with 10% ethanol in 10 mM acetic acid. The column is washed

with the same solution, and the peptide eluted with 50% ethanol in 10 mM acetic acid. The solvent may be removed by rotavaporation to yield the desalted peptide product.

Cyanogen Bromide Cleavage of Multicopy GLP Fragment.

The desalted multicopy GLP fragment may be dissolved in a 2M Citric Acid, pH 1.0 solution. Cyanogen bromide (CNBr) is added after purging the solution with Argon. After 4-5 hours of reaction the resulting pre-GLP fragments may be desalted using the procedure described above.

Cyanylation/Amidation of the Pre-GLP Fragments

The pre-GLP fragments may be dissolved in a pH 3.5 urea/ammonium acetate buffer and treated with excess DTT and DMAP-CN at room temperature for 15-30 minutes. The reaction mixture is then immediately desalted, e.g., using an low pressure C8 column or a Sephadex G-25 column. The desalted S-derivatized fragments are then dissolved in 3M aqueous ammonia and allowed to react for 30 minutes at room temperature to produce recombinant GLP1(7-36)-NH_2 (rGLP). The GLP1(7-36)-NH_2 (SEQ ID NO:53) may be further purified by HPLC on a semi-preparative C18 column using an acetonitrile gradient.

<u>Example 17. Production of GLP1(7-36)-NH_2 via CPD-Y Transamidation</u>

Amidated recombinant GLP1(7-36)-NH_2 (SEQ ID NO:53) may be prepared from a recombinant multicopy fusion peptide by cleavage, transamidation and photochemical rearrangement.

A first DNA construct is formed by joining four copies of the coding sequence for GLP1(7-36)-Met (SEQ ID NO:60) joined end to end. The DNA construct also has a nucleotide sequence coding for a methionine residue joined immediately upsteam of the DNA sequence encoding GLP1(7-36)-Met (SEQ ID NO:60). This DNA construct may be formed by automated DNA synthesis and subcloned into the <u>E. coli</u> expression vector pBN1. A second DNA

construct coding for a linking peptide which includes the thrombin cleavage site Gly-Pro-Arg is also subcloned into the resulting expression vector upstream from the first DNA construct. The expression vector may then be transformed into E. coli and the transformants selected and amplified. The multicopy fusion construct may be isolated as part of inclusion bodies from cell lysates as described in the Examples herein.

Treatment of the multicopy fusion construct with thrombin under the conditions described in Example 14 cleaves the hCAII peptide from the multi-GLP portion of the construct. The multicopy GLP peptide may be dissolved in 2M Citric Acid, pH 1.0 and cyanogen bromide (CNBr) added after purging the solution with Argon. The solution is permitted to react for 4-5 hours and the resulting fragments having the amino acid sequence GLP1(7-36)-Hse may be desalted on an low pressure C8 column.

The GLP1(7-36)-Hse (SEQ ID NO:61) fragments may be dissolved in 2 ml of 50 mM sodium carbonate buffer (pH 6.05) containing 1 mM EDTA and 250 mM (2-nitrophenyl)glycinamide (ONPGA). Reaction is initiated by the addition of a carboxypeptidase or a mutant of carboxypeptidase Y. The transamidation reaction provides the peptide GLP1(7-36)-ONPGA (SEQ ID NO:61) which upon irradiation with UV light of a wavelength no shorter than 320 nm is converted into GLP1(7-36)-NH_2 (SEQ ID NO:53).

Example 18. Production of GLP1(7-36)-NH_2 via CPD-Y Transpeptidation

The amidated recombinant peptide, GLP1(7-36)-NH_2, may also be prepared from a recombinant multicopy peptide by cleavage and transpeptidation.

The recombinant multicopy fusion peptide is produced by cells transformed with an expression vector. A first DNA construct is formed by joining four copies of the coding sequence for GLP1(7-35)-Met (SEQ ID NO:62)

end to end. The DNA construct also has a nucleotide sequence coding for a methionine residue joined immediately upsteam of the DNA sequences encoding GLP1(7-35)-Met (SEQ ID NO:62). This DNA construct may be formed by automated DNA synthesis and subcloned into the expression vector pBN1. A second DNA construct coding for a linking peptide which includes the thrombin cleavage site Gly-Pro-Arg is also subcloned into the expression vector upstream from the first DNA construct. The expression vector may then be transformed into E. coli and the transformants are selected and amplified. The multicopy fusion construct may be isolated as part of inclusion bodies from cell lysates as described in the Examples herein.

Treatment of the multicopy fusion construct with thrombin as described in Example 14 cleaves the hCAII peptide from the multi-GLP portion of the construct (a multicopy GLP1(7-35)-Met peptide). The multicopy GLP1(7-35)-Met peptide may be cleaved in citric acid solution containing cyanogen bromide into fragments having the amino acid sequence GLP1(7-35)-Hse (SEQ ID NO:54). After desalting on an low pressure C8 column, the fragments may be transpeptidated by treatment with carboxypeptidase Y in a sodium carbonate buffer (pH 9.5) containing EDTA and the amidated amino acid, Arg-NH_2. The transpeptidation reaction yields GLP1(7-36)-NH_2 (SEQ ID NO:53) which, if desired, may be further purified on a C8 HPLC column.

Example 19. Expression Plasmid for GLP1 Multicopy without hCAII

The following two synthetic DNA strands were prepared:

Oligo E: 5' A GGC GCC ATG GTC GGC GGC GGC GAC ATG CAT GCT GAA GG (SEQ ID NO:49)

Oligo F: 5′ CCT GGT CGA CTT ACT CGA GAG CGC AAC GAC CTT TAA
CCA GCC AAG CGA TGA ATT CTT TAG C
(SEQ ID NO:50)

The plasmid pBN4:GLP(7-36)C-1C was PCR-amplified using oligonucleotides E and F as primers. The PCR product was purified and digested with the restriction endonucleases Nco I and Xho I and ligated into the Nco I and Xho I digested expression vector pBN5. The resulting vector was designated pBN5:GLP(7-36)C-1C.

Multicopy GLP may be made by digestion of plasmid pBN5:GLP(7-36)C-x_c (where x represents the number of copies of the GLP sequence present in the plasmid) and purification of the DNA fragment containing the multicopy gene and and the T7 terminator sequence. The fragment is inserted into the digested plasmid pBN5:GLP(7-36)C-1C. The resulting expression vector is designated pBN5:GLP(7-36)C-(x+1)C, where x+1 represents the number of copies of the GLP sequence present in the plasmid.

Purification of the Recombinant Multicopy Polypeptides

A variety of methods for purification of a recombinant multicopy peptide are known in the art. Suitable purification methods are described, for example, in Kirshner et al., J. Biotechnology, 12:247-260 (1989) and Oldenburg et al., Prot.Expr.Purif., 5, 278 (1994), the disclosure of which is incorporated herein by reference.

Therapeutic Use of Recombinant Modified Polypeptide Products Produced by the Method of the Invention

The products of the present invention have significant therapeutic and supplemental physiological uses in clinical human and veterinary medical practice. For example, the insulinotrophic activity of GLP1(7-36)-NH_2 (SEQ ID NO:53) has been shown to be beneficial in

treating the symptoms of non-insulin dependent diabetes mellitus (NIDDM, Type II). Gutniak, New Eng. J. Med., 326:1316-2 (1992). GRF(1-44)-NH_2 (SEQ ID NO:57) is of therapeutic benefit for diseases such as short stature syndrome, endometriosis, and osteoporosis. In addition, supplemental GRF has been used to increase the lean to fat ratio in livestock allowing production of more wholesome meat products.

Methods of preparation of pharmaceutically functional compositions of the products of the invention, in combination with a physiologically acceptable carrier, are known in the art. A functional pharmaceutical composition must be administered in an effective amount, by known routes of administration. The dosage at which the functional pharmaceutical composition is applied is dependent on purpose for its use and the condition of the recipient.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, It will be apparent to one of ordinary skill in the art that many variations and modifications may be made while remaining within the spirit and scope of the invention.

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

Table 2 - Code Names for Plasmids

Plasmid Name	hCA	Mutations to hCA	Cloning Sites at C-terminus of hCA	Restriction Sites That Were Delted Other Than During Cloning Procedure
pA1	Yes	None	Hind III/EcoR V	-----
pA2	Yes	N10Q, N61Q, 6231A	Hind III/EcoR V	-----
pA3	Yes	None	EcoR I/Xho I	EcoR I from pBR 322
pBN1	Yes	None	Hind III/EcoR V	-----
pBN2	Yes	N10Q, N61Q, G231A	Hind III/EcoR V	-----
pBN3	Yes	None	Hind III/EcoR V	EcoR I from pBR 322
pBN4	Yes	None	EcoR I/Xho I	EcoR I from pBR3
pBN5	No	----	-----	-----
pA4	Yes	M240C	Hind III/EcoR V	-----
pA5	Yes	None	Hind III/Kpn I/ EcoR V	-----
pBN6	Yes	M240C	Hind III/EcoR V	------

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: BioNebraska, Inc.

(ii) TITLE OF THE INVENTION: PRODUCTION OF C-TERMINAL AMIDATED PEPTIDES FROM RECOMBINANT PROTEIN CS

(iii) NUMBER OF SEQUENCES: 62

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Merchant & Gould
(B) STREET: 3100 Norwest Center, 90 S. 7th Street
(C) CITY: Minneapolis
(D) STATE: MN
(E) COUNTRY: U.S.A.
(F) ZIP: 55402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 07-DEC-1995
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/350,528
(B) FILING DATE: 07-DEC-1994

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Carter, Charles G
(B) REGISTRATION NUMBER: 35,093
(C) REFERENCE/DOCKET NUMBER: 8648.43USWO

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: 612/332-9081
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 168 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...159
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATC AAA GCT TCT GCC ATG GGC GGC CGC GTC GAC CGT GGT CCG CGT TCT
  48
Ile Lys Ala Ser Ala Met Gly Gly Arg Val Asp Arg Gly Pro Arg Ser
 1               5                  10                  15

GTT TCT GAA ATC CAG CTG ATG CAC AAC CTG GGT AAA CAC CTG AAC TCT
  96
Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Asn Ser
            20                  25                  30

ATG GAA CGT GTT GAA TGG CTG CGT AAA AAA CTG CAG GAC GTT CAC AAC
 144
Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His Asn
        35                  40                  45

TTC TGC GCT CTC GAG TAAGATATC
  168
Phe Cys Ala Leu Glu
    50
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 1               5                  10                  15
Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Asn Ser
            20                  25                  30
Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His Asn
        35                  40                  45
Phe Cys Ala Leu Glu
    50
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 294 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...285

(D) OTHER INFORMATION:

(A) NAME/KEY: mat_peptide
(B) LOCATION: 1...0
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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ATC AAA GCT TCT GCC ATG GGC GGC CGC GTC GAC CGT GGT CCG CGT TCT
  48
Ile Lys Ala Ser Ala Met Gly Gly Arg Val Asp Arg Gly Pro Arg Ser
 1               5                  10                  15

GTT TCT GAA ATC CAG CTG ATG CAC AAC CTG GGT AAA CAC CTG AAC TCT
  96
Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Asn Ser
            20                  25                  30

ATG GAA CGT GTT GAA TGG CTG CGT AAA AAA CTG CAG GAC GTT CAC AAC
 144
Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His Asn
        35                  40                  45

TTC TGC GCT CTC GAC CGT GGT CCG GCT TCT GTT TCT GAA ATC CAG CTG
 192
Phe Cys Ala Leu Asp Arg Gly Pro Ala Ser Val Ser Glu Ile Gln Leu
    50                  55                  60

ATG CAC AAC CTG GGT AAA CAC CTG AAC TCT ATG GAA CGT GTT GAA TGG
 240
Met His Asn Leu Gly Lys His Leu Asn Ser Met Glu Arg Val Glu Trp
65                  70                  75                  80

CTG CGT AAA AAA CTG CAG GAC GTT CAC AAC TTC TGC GCT CTC GAG
TAAGAT    291
Leu Arg Lys Lys Leu Gln Asp Val His Asn Phe Cys Ala Leu Glu
                85                  90                  95

ATC
  294
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Ile Lys Ala Ser Ala Met Gly Gly Arg Val Asp Arg Gly Pro Arg Ser
 1               5                  10                  15
Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Asn Ser
            20                  25                  30
Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His Asn
        35                  40                  45
Phe Cys Ala Leu Asp Arg Gly Pro Ala Ser Val Ser Glu Ile Gln Leu
    50                  55                  60
```

```
Met His Asn Leu Gly Lys His Leu Asn Ser Met Glu Arg Val Glu Trp
65                  70                  75                  80
Leu Arg Lys Lys Leu Gln Asp Val His Asn Phe Cys Ala Leu Glu
                85                  90                  95
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 224 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...207
(D) OTHER INFORMATION:

(A) NAME/KEY: mat_peptide
(B) LOCATION: 1...0
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
AAA GCT TTC GGT GGT GGT GGT GGT CCG CGT GGT TGC GCC ATG GTC GAC
 48
Lys Ala Phe Gly Gly Gly Gly Gly Pro Arg Gly Cys Ala Met Val Asp
 1               5                  10                  15

GAC GAC GAC AAA TAC GCT GAC GCT ATC TTC ACC AAC TCT TAC CGT AAA
 96
Asp Asp Asp Lys Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys
            20                  25                  30

GTT CTG GGT CAG CTG TCT GCT CGT AAA CTG CTG CAG GAC ATC ATG TCC
 144
Val Leu Gly Gln Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser
        35                  40                  45

CGT CAG CAG GGT GAA TCT AAC CAG GAA CGT GGT GCT CGT GCT CGT CTG
 192
Arg Gln Gln Gly Glu Ser Asn Gln Glu Arg Gly Ala Arg Ala Arg Leu
    50                  55                  60

TGC GCT ATG CTC GAG TAAGATATCG AATTCGG
  224
Cys Ala Met Leu Glu
65
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Lys Ala Phe Gly Gly Gly Gly Gly Pro Arg Gly Cys Ala Met Val Asp
 1              5                  10                  15
Asp Asp Asp Lys Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys
            20                  25                  30
Val Leu Gly Gln Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser
        35                  40                  45
Arg Gln Gln Gly Glu Ser Asn Gln Glu Arg Gly Ala Arg Ala Arg Leu
    50                  55                  60
Cys Ala Met Leu Glu
65
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 369 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...366
(D) OTHER INFORMATION:

(A) NAME/KEY: mat_peptide
(B) LOCATION: 1...0
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
AAA GCT TTC GGT GGT GGT GGT GGT CCG CGT GGT TGC GCC ATG GTC GAC
  48
Lys Ala Phe Gly Gly Gly Gly Gly Pro Arg Gly Cys Ala Met Val Asp
 1              5                  10                  15

GAC GAC GAC AAA TAC GCT GAC GCT ATC TTC ACC AAC TCT TAC CGT AAA
  96
Asp Asp Asp Lys Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys
            20                  25                  30

GTT CTG GGT CAG CTG TCT GCT CGT AAA CTG CTG CAG GAC ATC ATG TCC
 144
Val Leu Gly Gln Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser
        35                  40                  45

CGT CAG CAG GGT GAA TCT AAC CAG GAA CGT GGT GCT CGT GCT CGT CTG
 192
Arg Gln Gln Gly Glu Ser Asn Gln Glu Arg Gly Ala Arg Ala Arg Leu
    50                  55                  60

TGC GCT ATG CTC GAC GAC GAC GAC AAA TAC GCT GAC GCT ATC TTC ACC
 240
```

```
Cys Ala Met Leu Asp Asp Asp Asp Lys Tyr Ala Asp Ala Ile Phe Thr
65                  70                  75                  80

AAC TCT TAC CGT AAA GTT CTG GGT CAG CTG TCT GCT CGT AAA CTG CTG
 288
Asn Ser Tyr Arg Lys Val Leu Gly Gln Leu Ser Ala Arg Lys Leu Leu
                85                  90                  95

CAG GAC ATC ATG TCC CGT CAG CAG GGT GAA TCT AAC CAG GAA CGT GGT
 336
Gln Asp Ile Met Ser Arg Gln Gln Gly Glu Ser Asn Gln Glu Arg Gly
            100                 105                 110

GCT CGT GCT CGT CTG TGC GCT ATG CTC GAG TAA
  369
Ala Arg Ala Arg Leu Cys Ala Met Leu Glu
        115                 120
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Lys Ala Phe Gly Gly Gly Gly Gly Pro Arg Gly Cys Ala Met Val Asp
1               5                   10                  15
Asp Asp Asp Lys Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys
            20                  25                  30
Val Leu Gly Gln Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser
        35                  40                  45
Arg Gln Gln Gly Glu Ser Asn Gln Glu Arg Gly Ala Arg Ala Arg Leu
    50                  55                  60
Cys Ala Met Leu Asp Asp Asp Asp Lys Tyr Ala Asp Ala Ile Phe Thr
65                  70                  75                  80
Asn Ser Tyr Arg Lys Val Leu Gly Gln Leu Ser Ala Arg Lys Leu Leu
                85                  90                  95
Gln Asp Ile Met Ser Arg Gln Gln Gly Glu Ser Asn Gln Glu Arg Gly
            100                 105                 110
Ala Arg Ala Arg Leu Cys Ala Met Leu Glu
        115                 120
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 174 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...165
(D) OTHER INFORMATION:

(A) NAME/KEY: mat_peptide
(B) LOCATION: 1...0
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
GAA TTT GGC GGC CGC GGT GGT GGT GGT GGT GTT AAC GGT CCG CGT GGT
  48
Glu Phe Gly Gly Arg Gly Gly Gly Gly Gly Val Asn Gly Pro Arg Gly
 1               5                  10                  15

TGC GCT GTC GAC ATG CAT GCT GAA GGT ACC TTC ACC TCC GAC GTT TCC
  96
Cys Ala Val Asp Met His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser
            20                  25                  30

TCC TAC CTG GAA GGT CAG GCT GCT AAA GAA TTC ATC GCT TGG CTG GTT
 144
Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val
        35                  40                  45

AAA GGT CGT TGC GCT CTC GAG TAAGTCGAC
  174
Lys Gly Arg Cys Ala Leu Glu
    50                  55
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Glu Phe Gly Gly Arg Gly Gly Gly Gly Gly Val Asn Gly Pro Arg Gly
 1               5                  10                  15
Cys Ala Val Asp Met His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser
            20                  25                  30
Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val
        35                  40                  45
Lys Gly Arg Cys Ala Leu Glu
    50                  55
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 279 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...270
(D) OTHER INFORMATION:

(A) NAME/KEY: mat_peptide
(B) LOCATION: 1...0
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
GAA TTT GGC GGC CGC GGT GGT GGT GGT GGT GTT AAC GGT CCG CGT GGT
  48
Glu Phe Gly Gly Arg Gly Gly Gly Gly Gly Val Asn Gly Pro Arg Gly
 1               5                  10                  15

TGC GCT GTC GAC ATG CAT GCT GAA GGT ACC TTC ACC TCC GAC GTT TCC
  96
Cys Ala Val Asp Met His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser
            20                  25                  30

TCC TAC CTG GAA GGT CAG GCT GCT AAA GAA TTC ATC GCT TGG CTG GTT
 144
Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val
        35                  40                  45

AAA GGT CGT TGC GCT CTC GAC ATG CAT GCT GAA GGT ACC TTC ACC TCC
 192
Lys Gly Arg Cys Ala Leu Asp Met His Ala Glu Gly Thr Phe Thr Ser
    50                  55                  60

GAC GTT TCC TCC TAC CTG GAA GGT CAG GCT GCT AAA GAA TTC ATC GCT
 240
Asp Val Ser Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala
65                  70                  75                  80

TGG CTG GTT AAA GGT CGT TGC GCT CTC GAG TAAGTCGAC
  279
Trp Leu Val Lys Gly Arg Cys Ala Leu Glu
                85                  90
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Glu Phe Gly Gly Arg Gly Gly Gly Gly Gly Val Asn Gly Pro Arg Gly
 1               5                  10                  15
```

```
Cys Ala Val Asp Met His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser
            20                  25                  30
Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val
        35                  40                  45
Lys Gly Arg Cys Ala Leu Asp Met His Ala Glu Gly Thr Phe Thr Ser
    50                  55                  60
Asp Val Ser Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala
65                  70                  75                  80
Trp Leu Val Lys Gly Arg Cys Ala Leu Glu
                85                  90
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...15
(D) OTHER INFORMATION:

(A) NAME/KEY: mat_peptide
(B) LOCATION: 1...0
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
GAC GAC GAC GAT AAA
   15 Asp Asp Asp Asp Lys    1                  5
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Asp Asp Asp Asp Lys
 1               5
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...12
(D) OTHER INFORMATION:

(A) NAME/KEY: mat_peptide
(B) LOCATION: 1...0
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATT GAA GGA AGA
12 Ile Glu Gly Arg 1

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ile Glu Gly Arg
1

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...24
(D) OTHER INFORMATION:

(A) NAME/KEY: mat_peptide
(B) LOCATION: 1...0
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CAT CCT TTT CAT CTG CTG GTT TAT
24 His Pro Phe His Leu Leu Val Tyr 1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

His Pro Phe His Leu Leu Val Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGCTTTCGTT GACGACGACG ATATCTT
27

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGCTAAGATA TCGTCGTCGT CAACGAA
27

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGCTGAATTC AACGTTCTCG AGGAT
25

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATCCTCGAGA ACGTTGAATT C
21

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AATCTAGAAA TAATTTTGTT TAACTTTAAG AAGG
34

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TAGAATTCCA TGGTATATCT CCTTCTTAAA
30

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCCAAGCTTC TGTTCGTGGT CCGCGTTCTG TTTCTGAAA
39

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GAAACAGAAC GCGGACCACG AACAGAAGCT TGGG
34

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCCAGCTGAT GCACAACCTG GGTAAACACC TGAACT
36

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AGGTGTTTAC CCAGGTTGTG CATCAGCTGG ATTTCA
36

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CTATGGAACG TGTTGAATGG CTGCGTAAAA AACTGCA
37

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TTTTTTACGC AGCCATTCAA CACGTTCCAT AGAGTTC
37

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGACGTTCAC AACTTCTAAG ATATCCGG
28

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CCGGATATCT TAGAAGTTGT GAACGTCCTG CAG
33

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TCAAAGCTTC TGCCATGGGC GGCCGCGTCG ACCGTGGTCC GCGTTCTGTT
TCTGAAATCC 60
AG
62

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CTCGATATCT TACTCGAGAG CGCAGAAGTT GTGAACGTCC
40

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Phe Val Asn Gly Pro Arg Ala Met Val Asp Asp Asp Asp Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GGCAAACACC AGGGACCTGA GCACTG
26

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CTGAGGATCC TCAACCAGGG TCATGCTTTC
30

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CTTAACTTCA ATGCGGAGGG TGAACC
26

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

TGCTGCAGGA CATCATGTCC CGTCAGCAGG GTGAATCTAA C
41

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CCGAATTCGA TATCTTACTC GAGCATAGCG CACAGACGAG CACGAGCACC
50

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CAAAGCTTTC GCCATGGTCG ACGACGACGA CAAATACGCT GACGCTATCT
TCACCAACTC 60
T
61

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GTCCTGCAGC AGTTTACGAG CAGACAGCTG ACCCAGAACT TTACGGTAAG
AGTTGGTGAA 60

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CCAAAGCTTT CGGTGGTGGT GGTGGTCCGC GTGGT
35

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GTCGTCGACC ATGGCGCAAC CACGCGGACC
30

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GTCAAATTTG GCGGCCGCGG TGGTGGTGGT GGTGTTAACG GTCCGCGTGG T
51

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTCCTCGAGG GTACCTTCAG CATGCATGTC GACAGCGCAA CCACGCGGAC CG
52

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CTGGGTACCT TCACCTCCGA CGTTTCCTCC TACCTGGAAG GTCAGGCTGC
TAAAGAATTC 60

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CCTGGTCGAC TTACTCGAGA GCGCAACGAC CTTTAACCAG CCAAGCGATG
AATTCTTTAG 60
C
61

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

AGGCGCCATG GTCGGCGGCG GCGACATGCA TGCTGAAGG
39

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CCTGGTCGAC TTACTCGAGA GCGCAACGAC CTTTAACCAG CCAAGCGATG
AATTCTTTAG 60
C
61

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

```
His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val
 1               5                  10                  15
Ser Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu
            20                  25                  30
Val Lys Gly Arg
        35
```

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

```
His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
 1               5                  10                  15
Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

```
His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
 1               5                  10                  15
Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg
            20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

```
His Asp Glu Phe Glu Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val
 1               5                  10                  15
Ser Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu
            20                  25                  30
Val Lys Gly Xaa
        35
```

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

```
Ala Met His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu
 1               5                  10                  15
Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg
            20                  25                  30
Cys
```

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

```
Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Asn
 1               5                  10                  15
Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His
            20                  25                  30
Asn Phe
```

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

```
Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly Gln
 1               5                  10                  15
Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Arg Gln Gln Gly
            20                  25                  30
Glu Ser Asn Gln Glu Arg Gly Ala Arg Ala Arg Leu
        35                  40                  45
```

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

```
Asp Asp Asp Asp Lys Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg
 1               5                  10                  15
Lys Val Leu Gly Gln Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met
            20                  25                  30
Ser Arg Gln Gln Gly Glu Ser Asn Gln Glu Arg Gly Ala Arg Ala Arg
        35                  40                  45
Leu
    50
```

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

```
Gly Cys Ala Val Asp Met
 1               5
```

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

```
His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
 1               5                  10                  15
Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Met
            20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1 5 10 15
Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Xaa
20 25 30

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1 5 10 15
Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Met
20 25 30

WHAT IS CLAIMED IS:

1. A method of producing a peptide having a C-terminal α-carboxamide comprising:

converting a recombinant protein construct to a product peptide having a C-terminal α-carboxamide; wherein,

the recombinant protein construct has an amino acid sequence of the formula:

$$\text{Yyy-TargP'-(CS2)-[-(Ln1)}_n\text{-(CS1)}_m\text{-TargP-(CS2)-]}_r\text{- Xxx}$$

-CS1- is a cleavage site;
-CS2- cleavage site is a methionine residue or an unblocked cysteine residue;
-(Ln1)- is a linking peptide;
-TargP- and -TargP'- are a target peptide which is free of at least one amino acid residue selected from the group consisting of a methionine residue and an unblocked cysteine residue;
n and m are 0 or 1;
r is an integer from 1 to about 150;
Yyy- is a leader group; and
-Xxx is a tail group.

2. The method of claim 1 further comprising isolating an inclusion body which includes the recombinant protein construct.

3. The method of claim 1 wherein Yyy- comprises an adjunct peptide.

4. The method of claim 3 wherein the adjunct peptide includes a cleavage site connected to the N-terminus of -TargP'-.

5. The method of claim 3 wherein the adjunct peptide includes a fragment selected from the group

consisting of a ligand binding protein, a highly charged peptide, an antigenic peptide, a polyhistidine-containing peptide, a hydrophobic peptide and a DNA binding peptide.

6. The method of claim 5 wherein the ligand binding protein includes a carbonic anhydrase.

7. The method of claim 1 wherein n is 1 and Ln1 includes a second target peptide.

8. The method of claim 1 wherein the target peptide is free of unblocked cysteine residues; -(CS2)- is a cysteine residue; the step of converting includes reacting -(CS2)- with an S-cyanylating agent to form an S-derivatized -(CS2)-; and reacting the S-derivatized -(CS2)- with an amino compound to produce the product peptide.

9. The method of claim 8 wherein the S-cyanylating agent includes a thiocyanato substituted aromatic compound or a 1-cyano-4-(dialkylamino)pyridinium salt.

10. The method of claim 9 wherein the thiocyanato substituted aromatic compound includes 2-nitro-5-thiocyanatobenzoic acid or a salt thereof.

11. The method of claim 9 wherein the 1-cyano-4-(dialkylamino)pyridinium salt includes 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate, 1-cyano-4-(N-methyl,N-benzylamino)pyridinium tetrafluoroborate or 1-cyano-4-(pyrrolidino)-pyridinium tetrafluoroborate.

12. The method of claim 8 wherein the target peptide includes an amino acid sequence corresponding to a

peptide selected from the group consisting of GLP1(7-35) (SEQ ID NO:52), GRF(1-44) (SEQ ID NO:57), PTH(1-34) (SEQ ID NO:56) and substance P.

13. The method of claim 8 wherein the target peptide is free of methionine residues.

14. The method of claim 13 wherein m is 1; the -(CS1)- cleavage site is a methionine residue; and the step of converting includes contacting the -(CS1)- cleavage site with cyanogen bromide to cleave the C-terminal peptide bond of the methionine residue.

15. The method of claim 14 wherein Yyy- includes an adjunct peptide having a methionine residue connected to the N-terminus of -TargP'-.

16. The method of claim 13 wherein the target peptide includes an amino acid sequence corresponding to GLP1(7-35) (SEQ ID NO:52).

17. The method of claim 8 wherein the target peptide includes a methionine residue; m is 1; the -(CS1)- cleavage site is free of unblocked cysteine residues; and the step of converting includes cleaving at the -(CS1)- cleavage site and the cleaving step does not include contacting the -(CS1)- cleavage site with cyanogen bromide.

18. The method of claim 17 wherein the -(CS1)- cleavage site is an enzymatic cleavage site recognized by an endopeptidase.

19. The method of claim 18 wherein the cleaving step includes contacting the -(CS1)- cleavage site with an endopeptidase selected from the group consisting

of enterokinase, Factor Xa, ubiquitin cleaving enzyme, thrombin, trypsin, renin, subtilisin, chymotrypsin, clostripain, papain, ficin, and *S. aureus* V8.

20. The method of claim 18 wherein the step of converting includes contacting the -(CS1)- cleavage site with an endopeptidase to produce an intermediate peptide which includes -(CS2)-; contacting the intermediate peptide with the S-cyanylating agent to form an S-derivatized -(CS2)-; and reacting the S-derivatized -(CS2)- with the amino compound to produce the product peptide.

21. The method of claim 18 wherein the step of converting includes contacting the -(CS2)- with the S-cyanylating agent to form an S-derivatized -(CS2)-; reacting the S-derivatized -(CS2)- with the amino compound to form an intermediate peptide which includes the -(CS1)- cleavage site; and contacting the intermediate peptide with the endopeptidase for -(CS1)- to produce the product peptide.

22. The method of claim 18 wherein the target peptide includes an amino acid sequence corresponding to a peptide selected from the group consisting of GRF(1-44) (SEQ ID NO:57) and PTH(1-34) (SEQ ID NO:56).

23. The method of claim 1 wherein the target peptide is free of unblocked cysteine residues; -(CS2)- is a cysteine residue; and the step of converting includes reacting -(CS2)- with an S-cyanylating agent to form an S-derivatized -(CS2)-; and reacting the S-derivatized -(CS2)- with hydroxide to produce a precursor peptide.

24. The method of claim 23 wherein the step of converting further comprises transpeptidating the precursor peptide with an amidated amino acid in the presence of an exopeptidase to produce a C-terminal amidated peptide.

25. The method of claim 23 wherein the precursor peptide includes a C-terminal glycine residue; and the step of converting further comprises decomposing the glycine residue in the presence of a glycine monooxygenase to produce a C-terminal amidated peptide.

26. The method of claim 23 wherein the step of converting further comprises transamidating the precursor peptide with a 2-nitrobenzylamine compound in the presence of a carboxypeptidase to replace the C-terminal precursor peptide residue with a C-terminal (2-nitrobenzyl)amido group; and photochemically decomposing the (2-nitrobenzyl)amido group to produce a C-terminal amidated peptide.

27. The method of claim 1 wherein the step of converting includes cleaving the recombinant protein construct at -(CS2)- to produce a first peptide and modifying the first peptide to produce the product peptide.

28. The method of claim 27 wherein the target peptide is free of methionine residues; the -(CS2)- cleavage site is a methionine residue; and

wherein cleaving step includes treating the recombinant protein construct with cyanogen bromide to produce a first peptide having a C-terminal homoserine residue; and treating the first peptide with a carboxypeptidase in the presence of an amino

acid having a C-terminal α-carboxamide to produce the product peptide.

29. The method of claim 28 wherein the target peptide includes an amino acid sequence corresponding to a peptide selected from the group consisting of calcitonin and GLP1(7-35) (SEQ ID NO:52).

30. The method of claim 28 wherein n and m are 0.

31. The method of claim 28 wherein the carboxypeptidase includes carboxypeptidase Y.

32. The method of claim 28 wherein the carboxypeptidase includes a mutant of carboxypeptidase Y capable of transpeptidating the C-terminal residue of a peptide with an arginine α-amide.

33. A method of producing a peptide having a C-terminal α-carboxamide comprising:
i) expressing a recombinant protein construct in a host cell, wherein the recombinant protein construct includes an amino acid sequence having the formula:

$$\text{Yyy-TargP-(CS2)-[-(Ln1)}_n\text{-(CS1)}_m\text{-TargP-(CS2)-]}_r\text{-Xxx}$$

-CS1- is a cleavage site;
-CS2- cleavage site is a methionine residue or an unblocked cysteine residue;
-(Ln1)- is a linking peptide;
-TargP- is a target peptide which is free of at least one amino acid residue selected from the group consisting of a methionine residue and an unblocked cysteine residue;
n and m are 0 or 1;
r is an integer from 1 to about 150;

Yyy- is a leader group; and
-Xxx is a tail group;
ii) isolating the recombinant protein construct; and
iii) converting the recombinant protein construct to a product peptide having a C-terminal α-carboxamide.

34. A recombinant protein construct comprising an amino acid sequence of the formula:

Yyy-(CS1)-TargP-(Cys)-Xxx

wherein Yyy- is a leader group which includes an amino acid residue;
-(CS1)- is a cleavage site which is free of unblocked cysteine residues;
-TargP- is a target peptide which is free of unblocked cysteine residues; and
-Xxx is a tail group which includes an amino acid residue.

35. The method of claim 34 wherein Yyy- comprises a binding protein.

36. A recombinant protein construct having the formula:

$$\text{Yyy-TargP-(CS2)-[-(Ln1)}_n\text{-(CS1)}_m\text{-TargP-(CS2)-]}_r\text{-Xxx}$$

-CS1- is a cleavage site;
-CS2- cleavage site is a methionine residue or an unblocked cysteine residue;
-(Ln1)- is a linking peptide;
-TargP- is a target peptide which is free of at least one amino acid residue selected from the group consisting of a methionine residue and an unblocked cysteine residue;

n and m are 0 or 1;
r is an integer from 1 to about 150;
Yyy- is a leader group; and
-Xxx is a tail group.

37. A recombinant gene containing a DNA sequence coding for a peptide which includes an amino acid sequence having the formula:

$$\text{Yyy-TargP-(CS2)-[-(Ln1)}_n\text{-(CS1)}_m\text{-TargP-(CS2)-]}_r\text{-Xxx}$$

-CS1- is a cleavage site;
-CS2- cleavage site is a methionine residue or an unblocked cysteine residue;
-(Ln1)- is a linking peptide;
-TargP- is a target peptide which is free of at least one amino acid residue selected from the group consisting of a methionine residue and an unblocked cysteine residue;
n and m are 0 or 1;
r is an integer from 1 to about 150;
Yyy- is a leader group; and
-Xxx is a tail group.

38. An expression cassette comprising a nucleic acid sequence coding for a peptide which includes an amino acid sequence of the formula:

$$\text{Yyy-TargP-(CS2)-[-(Ln1)}_n\text{-(CS1)}_m\text{-TargP-(CS2)-]}_r\text{-Xxx}$$

-CS1- is a cleavage site;
-CS2- cleavage site is a methionine residue or an unblocked cysteine residue;
-(Ln1)- is a linking peptide;
-TargP- is a target peptide which is free of at least one amino acid residue selected from the group consisting of a methionine residue and an unblocked cysteine residue;

n and m are 0 or 1;
r is an integer from 1 to about 150;
Yyy- is a leader group; and
-Xxx is a tail group; and

wherein the nucleic acid sequence coding for the peptide is operably linked to a promoter functional in a vector.

39. An expression vector comprising the expression cassette of claim 38.

40. A transformed cell comprising a recombinant gene including a DNA sequence coding for a peptide which includes an amino acid sequence having the formula:

$$\text{Yyy-TargP-(CS2)-[-(Ln1)}_n\text{-(CS1)}_m\text{-TargP-(CS2)-]}_r\text{-Xxx}$$

-CS1- is a cleavage site;
-CS2- cleavage site is a methionine residue or an unblocked cysteine residue;
-(Ln1)- is a linking peptide;
-TargP- is a target peptide which is free of at least one amino acid residue selected from the group consisting of a methionine residue and an unblocked cysteine residue;
n and m are 0 or 1;
r is an integer from 1 to about 150;
Yyy- is a leader group; and
-Xxx is a tail group.

FIG. 1

```
                                                                  THROMBIN   PTH(1-
-hCA
Ile  Lys  Ala  Ser  Ala  Met  Gly  Gly  Arg  Val  Asp  Arg  Gly  Pro  Arg  Ser  Val  Ser
ATC  AAA  GCT  TCT  GCC  ATG  GGC  GGC  CGC  GTC  GAC  CGT  GGT  CCG  CGT  TCT  GTT  TCT
TAG  TTT  CGA  AGA  CGG  TAC  CCG  CCG  GCG  CAG  CTG  GCA  CCA  GGC  GCA  AGA  CAA  AGA
      Hind III      Nco I      NotI           Sal I

Glu  Ile  Gln  Leu  Met  His  Asn  Leu  Gly  Lys  His  Leu  Asn  Ser  Met  Glu  Arg  Val
GAA  ATC  CAG  CTG  ATG  CAC  AAC  CTG  GGT  AAA  CAC  CTG  AAC  TCT  ATG  GAA  CGT  GTT
CTT  TAG  GTC  GAC  TAC  GTG  TTG  GAC  CCA  TTT  GTG  GAC  TTG  AGA  TAC  CTT  GCA  CAA

                                             34) CYS
Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His Asn Phe Cys Ala Leu Glu Stop
GAA TGG CTG CGT AAA AAA CTG CAG GAC GTT CAC AAC TTC TGC GCT CTC GAG TAA GAT ATC
CTT ACC GAG GCA TTT TTT GAC GTC CTG CAA GTG TTG AAG ACG CGA GAG CTC ATT CTA TAG
                        Pst I                               Xho I       EcoR V
```

FIG. 2

```
-hCA                                                                THROMBIN    PTH(1-
Ile  Lys  Ala  Ser  Ala  Met  Gly  Gly  Arg  Val  Asp  Arg  Gly  Pro  Arg  Ser  Val  Ser
ATC  AAA  GCT  TCT  GCC  ATG  GGC  GGC  CGC  GTC  GAC  CGT  GGT  CCG  CGT  TCT  GTT  TCT
TAG  TTT  CGA  AGA  CGG  TAC  CCG  CCG  GCG  CAG  CTG  GCA  CCA  GGC  GCA  AGA  CAA  AGA
       Hind III      Nco I    NotI           Sal I

Glu  Ile  Gln  Leu  Met  His  Asn  Leu  Gly  Lys  His  Leu  Asn  Ser  Met  Glu  Arg  Val
GAA  ATC  CAG  CTG  ATG  CAC  AAC  CTG  GGT  AAA  CAC  CTG  AAC  TCT  ATG  GAA  CGT  GTT
CTT  TAG  GTC  GAC  TAC  GTG  TTG  GAC  CCA  TTT  GTG  GAC  TTG  AGA  TAC  CTT  GCA  CAA

                                                       34)  CYS
Glu  Trp  Leu  Arg  Lys  Lys  Leu  Gln  Asp  Val  His  Asn  Phe  Cys  Ala  Leu  Asp  Arg  Gly
GAA  TGG  CTG  CGT  AAA  AAA  CTG  CAG  GAC  GTT  CAC  AAC  TTC  TGC  GCT  CTC  GAC  CGT  GGT
CTT  ACC  GAG  GCA  TTT  TTT  GAC  GTC  CTG  CAA  GTG  TTG  AAG  ACG  CGA  GAG  CTG  GCA  CCA
                               Pst I

THROMBIN   PTH(1-
Pro  Arg  Ser  Val  Ser  Glu  Ile  Gln  Leu  Met  His  Asn  Leu  Gly  Lys  His  Leu  Asn
CCG  CGT  TCT  GTT  TCT  GAA  ATC  CAG  CTG  ATG  CAC  AAC  CTG  GGT  AAA  CAC  CTG  AAC
GGC  GCA  AGA  CAA  AGA  CTT  TAG  GTC  GAC  TAC  GTG  TTG  GAC  CCA  TTT  GTG  GAC  TTG

Ser  Met  Glu  Arg  Val  Glu  Trp  Leu  Arg  Lys  Lys  Leu  Gln  Asp  Val  His  Asn
TCT  ATG  GAA  CGT  GTT  GAA  TGG  CTG  CGT  AAA  AAA  CTG  CAG  GAC  GTT  CAC  AAC
AGA  TAC  CTT  GCA  CAA  CTT  ACC  GAG  GCA  TTT  TTT  GAC  GTC  CTG  CAA  GTG  TTG
                                                    Pst I

34)  CYS
Phe  Cys  Ala  Leu  Glu  Stop
TTC  TGC  GCT  CTC  GAG  TAA  GAT  ATC
AAG  ACG  CGA  GAG  CTC  ATT  CTA  TAG
                 Xho I        EcoR V
```

FIG. 3

-hCA								THROMBIN			CYS						ENTERO-
Lys	Ala	Phe	Gly	Gly	Gly	Gly	Gly	Pro	Arg	Gly	Cys	Ala	Met	Val	Asp	Asp	Asp
AAA	GCT	TTC	GGT	GGT	GGT	GGT	GGT	CCG	CGT	GGT	TGC	GCC	ATG	GTC	GAC	GAC	GAC
TTT	CGA	AAG	CCA	CCA	CCA	CCA	CCA	GGC	GCA	CCA	ACG	CGG	TAC	CAG	CTG	CTG	CTG
HindIII												(NcoI)	SalI				

KINASE		GRF															
		1	2	3	4	5*	6	7	8	9	10	11	12	13*	14*	15	16
Asp	Lys	Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Gly	Gln
GAC	AAA	TAC	GCT	GAC	GCT	ATC	TTC	ACC	AAC	TCT	TAC	CGT	AAA	GTT	CTG	GGT	CAG
CTG	TTT	ATG	CGA	CTG	CGA	TAG	AAG	TGG	TTG	AGA	ATG	GCA	TTT	CAA	GAC	CCA	GTC
						(SspI)									(AvrII)		PvuII

17	18	19	20	21	22	23	24	25	26	27	28*	29*	30	31	32	33	34
Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Met	Ser	Arg	Gln	Gln	Gly	Glu	Ser
CTG	TCT	GCT	CGT	AAA	CTG	CTG	CAG	GAC	ATC	ATG	TCC	CGT	CAG	CAG	GGT	GAA	TCT
GAC	AGA	CGA	GCA	TTT	GAC	GAC	GTC	CTG	TAG	TAC	AGG	GCA	GTC	GTC	CCA	CTT	AGA
						PstI						(SalI)					

35	36	37	38	39*	40	41	42	43	44	CYS					
Asn	Gln	Glu	Arg	Gly	Ala	Arg	Ala	Arg	Leu	Cys	Ala	Met	Leu	Glu	Stop
AAC	CAG	GAA	CGT	GGT	GCT	CGT	GCT	CGT	CTG	TGC	GCT	ATG	CTC	GAG	TAA
TTG	GTC	CTT	GCA	CCA	CGA	GCA	CGA	GCA	GAC	ACG	CGA	TAC	GAG	CTC	ATT
					(SacI)								XhoI		

GAT	ATC	GAA	TTC	GG
CTA	TAG	CTT	AAG	CC
EcoRV		EcoRI		

FIG. 4A

-hCA								THROMBIN			CYS						ENTEROKINASE	
Lys	Ala	Phe	Gly	Gly	Gly	Gly	Gly	Pro	Arg	Gly	Cys	Ala	Met	Val	Asp	Asp	Asp	
AAA	GCT	TTC	GGT	GGT	GGT	GGT	GGT	CCG	CGT	GGT	TGC	GCC	ATG	GTC	GAC	GAC	GAC	
TTT	CGA	AAG	CCA	CCA	CCA	CCA	CCA	GGC	GCA	CCA	ACG	CGG	TAC	CAG	CTG	CTG	CTG	
	HindIII												(Nco I)			Sal I		

	GRF	1	2	3	4	5*	6	7	8	9	10	11	12	13	14	15	16
Asp	Lys	Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Gly	Gln
GAC	AAA	TAC	GCT	GAC	GCT	ATC	TTC	ACC	AAC	TCT	TAC	CGT	AAA	GTT	CTG	GGT	CAG
CTG	TTT	ATG	CGA	CTG	CGA	TAG	AAG	TGG	TTG	AGA	ATG	GCA	TTT	CAA	GAC	CCA	GTC
						(Ssp I)									(AvrII)		PvuII

17	18	19	20	21	22	23	24	25	26	27	28*	29*	30	31	32	33	34
Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Met	Ser	Arg	Gln	Gln	Gly	Glu	Ser
CTG	TCT	GCT	CGT	AAA	CTG	CTG	CAG	GAC	ATC	ATG	TCC	CGT	CAG	CAG	GGT	GAA	TCT
GAC	AGA	CGA	GCA	TTT	GAC	GAC	GTC	CTG	TAG	TAC	AGG	GCA	GTC	GTC	CCA	CTT	AGA
						Pst I						(Sal I)					

35	36	37	38	39*	40	41	42	43	44	CYS				ENTEROKINASE		
Asn	Gln	Glu	Arg	Gly	Ala	Arg	Ala	Arg	Leu	Cys	Ala	Met	Leu	Asp	Asp	Asp
AAC	CAG	GAA	CGT	GGT	GCT	CGT	GCT	CGT	CTG	TGC	GCT	ATG	CTC	GAC	GAC	GAC
TTG	GTC	CTT	GCA	CCA	CGA	GCA	CGA	GCA	GAC	ACG	CGA	TAC	GAG	CTG	CTG	CTG
				(Sac I)												

FIG. 4B

	GRF	1	2	3	4	5*	6	7	8	9	10	11	12	13	14	15	16
Asp	Lys	Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Gly	Gln
GAC	AAA	TAC	GCT	GAC	GCT	ATC	TTC	ACC	AAC	TCT	TAC	CGT	AAA	GTT	CTG	GGT	CAG
CTG	TTT	ATG	CGA	CTG	CGA	TAG	AAG	TGG	TTG	AGA	ATG	GCA	TTT	CAA	GAC	CCA	GTC
						(Ssp I)									(AvrII)		PvuII

17	18	19	20	21	22	23	24	25	26	27	28*	29*	30	31	32	33	34
Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Met	Ser	Arg	Gln	Gln	Gly	Glu	Ser
CTG	TCT	GCT	CGT	AAA	CTG	CTG	CAG	GAC	ATC	ATG	TCC	CGT	CAG	CAG	GGT	GAA	TCT
GAC	AGA	CGA	GCA	TTT	GAC	GAC	GTC	CTG	TAG	TAC	AGG	GCA	GTC	GTC	CCA	CTT	AGA
						Pst I					(Sal I)						

35	36	37	38	39*	40	41	42	43	44	CYS					
Asn	Gln	Glu	Arg	Gly	Ala	Arg	Ala	Arg	Leu	Cys	Ala	Met	Leu	Glu	Stop
AAC	CAG	GAA	CGT	GGT	GCT	CGT	GCT	CGT	CTG	TGC	GCT	ATG	CTC	GAG	TAA
TTG	GTC	CTT	GCA	CCA	CGA	GCA	CGA	GCA	GAC	ACG	CGA	TAC	GAG	CTC	ATT
				(Sac I)									Xho I		

FIG. 5

```
-hCAII                                                     THROMBIN            C/A
Glu Phe Gly Gly Arg Gly Gly Gly Gly Gly Val Asn Gly Pro Arg Gly Cys Ala Val Asp
GAA TTT GGC GGC CGC GGT GGT GGT GGT GGT GTT AAC GGT CCG CGT GGT TGC GCT GTC GAC
CTT AAA CCG CCG GCG CCA CCA CCA CCA CCA CAA TTG CCA GGC GCA CCA ACG CGA CAG CTG
 ApoI      NotI                            HpaI                            SalI

    GLP1
CNBr7  8   9   10  11  12  13  14  15  16  17  18  19  20  21  22  23  24  25
Met His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly Gln Ala Ala
ATG CAT GCT GAA GGT ACC TTC ACC TCC GAC GTT TCC TCC TAC CTG GAA GGT CAG GCT GCT
TAC GTA CGA CTT CCA TGG AAG TGG AGG CTG CAA AGG AGG ATG GAC CTT CCA GTC CGA CGA
 NsiI              KpnI
    SphI

26  27  28  29  30  31  32  33  34  35  36      C/A
Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Cys Ala Leu Glu Stop
AAA GAA TTC ATC GCT TGG CTG GTT AAA GGT CGT TGC GCT CTC GAG TAA GTC GAC
TTT CTT AAG TAG CGA ACC GAC CAA TTT CCA GCA ACG CGA GAG CTC ATT CAG CTG
     EcoRI                                          XhoI         SalI
```

FIG. 6A

```
-hCAII                                                            THROMBIN        C/A
Glu Phe Gly Gly Arg Gly Gly Gly Gly Gly Val Asn Gly Pro Arg Gly Cys Ala Val Asp
GAA TTT GGC GGC CGC GGT GGT GGT GGT GGT GTT AAC GGT CCG CGT GGT TGC GCT GTC GAC
CTT AAA CCG CCG GCG CCA CCA CCA CCA CCA CAA TTG CCA GGC GCA CCA ACG CGA CAG CTG
ApoI     NotI                           HpaI                            SalI

    GLP1
CNBr 7   8   9   10  11  12  13  14  15  16  17  18  19  20  21  22  23  24  25
Met His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly Gln Ala Ala
ATG CAT GCT GAA GGT ACC TTC ACC TCC GAC GTT TCC TCC TAC CTG GAA GGT CAG GCT GCT
TAC GTA CGA CTT CCA TGG AAG TGG AGG CTG CAA AGG AGG ATG GAC CTT CCA GTC CGA CGA
 NsiI           KpnI
   SphI

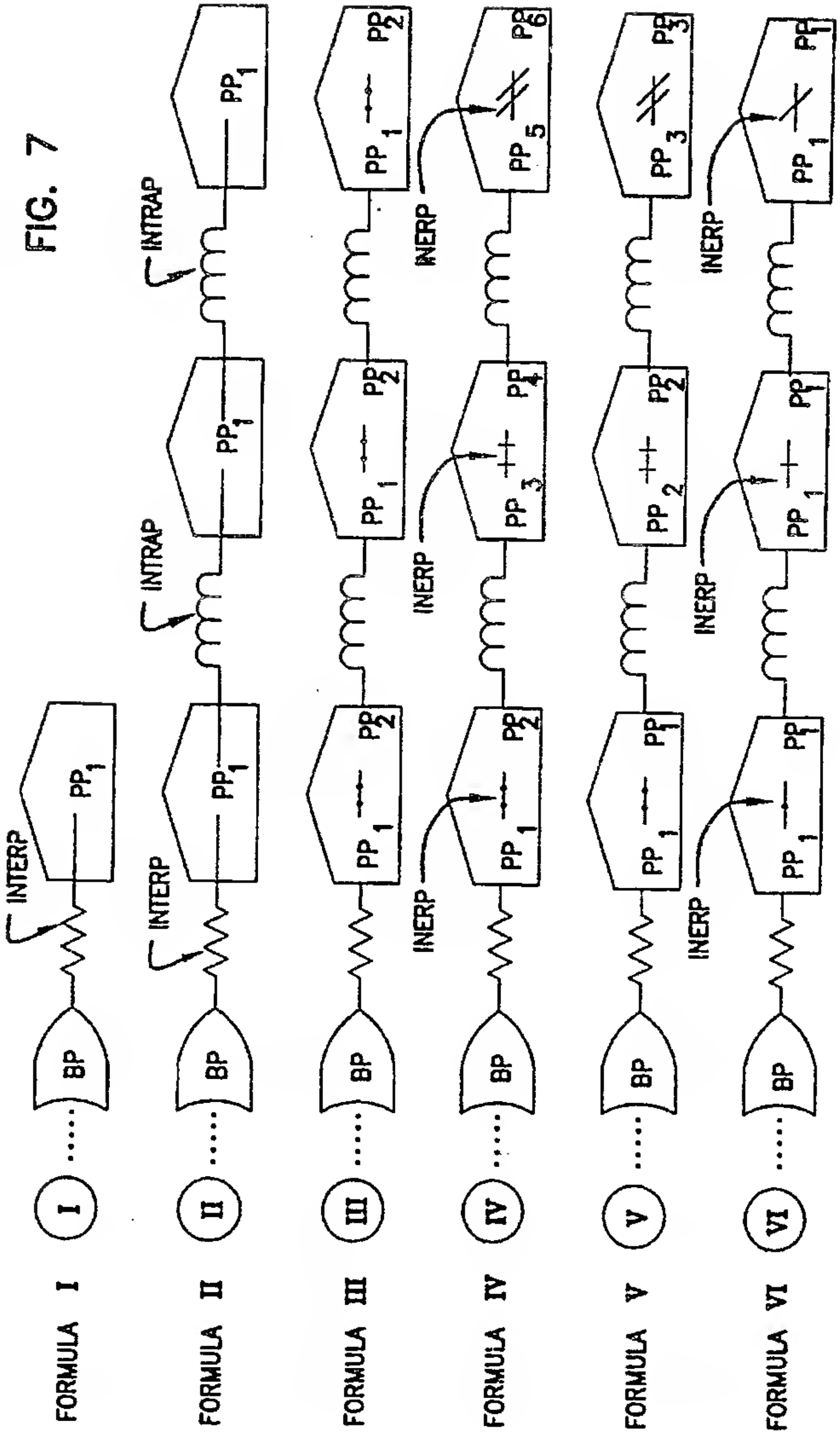
                                                                  GLP1
26  27  28  29  30  31  32  33  34  35  36  C/A             CNBr7   8   9   10
Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Cys Ala Leu Asp Met His Ala Glu Gly
AAA GAA TTC ATC GCT TGG CTG GTT AAA GGT CGT TGC GCT CTC GAC ATG CAT GCT GAA GGT
TTT CTT AAG TAG CGA ACC GAC CAA TTT CCA GCA ACG CGA GAG CTG TAC GTA CGA CTT CCA
     EcoRI                                                  NsiI            KpnI
                                                              SphI
```

FIG. 6B

```
11  12  13  14  15  16  17  18  19  20  21  22  23  24  25  26  27  28  29  30
Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala
ACC TTC ACC TCC GAC GTT TCC TCC TAC CTG GAA GGT CAG GCT GCT AAA GAA TTC ATC GCT
TGG AAG TGG AGG CTG CAA AGG AGG ATG GAC CTT CCA GTC CGA CGA TTT CTT AAG TAG CGA
                                                                  EcoRI

31  32  33  34  35  36    C/A
Trp Leu Val Lys Gly Arg Cys Ala Leu Glu Stop
TGG CTG GTT AAA GGT CGT TGC GCT CTC GAG TAA GTC GAC
ACC GAC CAA TTT CCA GCA ACG CGA GAG CTC ATT CAG CTG
                                  XhoI      (Sal I)
```

FIG. 7


FORMULA I
FORMULA II
FORMULA III
FORMULA IV
FORMULA V
FORMULA VI
BP
INTERP
INTRAP
INERP
PP1
PP2
PP3
PP4
PP5
PP6